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The Yeast Nucleoporin Nup53p Provides a Specific Binding Site for the Karyopherin Kap121p and Has a Role in Nuclear Pore Complex Assembly

by

Marcello Marelli ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment
of the requirements for the degree of Doctor of Philosophy

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Marcello Marelli

Marcello Marelli
Strada prov. di Montefallonio
Peveragno (CN) 12016
Italy

31 August 2000

Abstract

We have identified a subcomplex of the nuclear pore complex (NPC) that acts as a specific binding site for the karyopherin Kap121p. This subcomplex contains two previously uncharacterized and structurally related nucleoporins, Nup53p and Nup59p, and the NPC core protein Nup170p. The localization of Nup53p, Nup59p, and Nup170p within the NPC was defined by immunoelectron microscopy and suggests that this subcomplex is positioned on both cytoplasmic and nucleoplasmic sides of the NPC. The karyopherin Kap121p was also found in association with the isolated NPC subcomplex. *In vitro* binding assays showed that each of the nucleoporins interacts with the others, but the interaction of the subcomplex with Kap121p is mediated directly by Nup53p. Mutations in *NUP53* affect the subcellular distribution of Kap121p as well as the Kap121p-mediated nuclear import of a ribosomal L25-reporter protein. Interestingly, Nup53p was found to be phosphorylated specifically in mitosis. This phenomenon correlates with a transient decrease in perinuclear-associated Kap121p. We have also investigated the functions of Nup53p in nuclear transport and its role in the formation of nuclear pores. By examining the effects of altering *NUP53* expression, we showed that overproduction of Nup53p inhibited the import of Pho4p, a Kap121p substrate, but not of a Kap95p-dependent import cargo. The Nup53p produced by overexpression of its gene was visualized by immunofluorescence microscopy and shown to accumulate at the nuclear envelope in a continuous, nuclear rim-staining pattern distinct from the punctate distribution seen at wild type levels. Strikingly, overproduction of Nup53p induced the proliferation of intranuclear membrane-enclosed cisternae that appear to originate from the inner nuclear membrane. These structures formed multiple layers of double

membrane lamellae generally positioned adjacent to the nuclear envelope. Like the nuclear envelope, these lamellar membranes contained transcisternal pores and two integral membrane proteins, Pom152 and Ndc1p, but lacked fully assembled NPCs. Interestingly, both Kap121p and Nup170p are essential for the nuclear localization of Nup53p and the proliferation of intranuclear membranes. Moreover, Kap121p and Nup170p were found to be required for the proper assembly of endogenous Nup53p within the NPCs.

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: **The yeast nucleoporin Nup53p provides a specific binding site for the karyopherin Kap121p and has a role in NPC assembly**, submitted by Marcello Marelli in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Richard W. Wozniak

Richard W. Wozniak, Ph.D.

B. Burke

Brian E. Burke, Ph.D.

Richard A. Rachubinski, Ph.D.

Frank Nargang

Frank E. Nargang, Ph.D.

Paul R. Melançon, Ph.D.

31 August 2000

Date

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List of Abbreviations and Symbols

5-FOA	5'-fluoroorotic acid
ADP	adenosine diphosphate
ARF	ADP-ribosylation factors
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
cNLS	'classical'nuclear localization signal
CoA	coenzyme A
D	Dalton
DAPI	diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
ECL	enhanced chemoluminescence
ER	endoplasmic reticulum
F	farad
FEISEM	field emission in-lens scanning electron microscopy
FRET	fluorescence resonance energy transfer
g	gram
g	gravitational force
GDP	guanosine diphosphate
GFP	green fluorescent protein
GST	glutathione-S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
GTPγS	non-hydrolyzable analogue of GTP
h	hour
hnRNP	heterogeneous nuclear RNP
HRP	horseradish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactoside
Kap	karyopherin
M	moles per litre or Mega (1×10^6)
mRNA	messenger RNA
NE	nuclear envelope
NES	nuclear export signal
NLS	nuclear loclaization signal
NPC	nuclear pore complex
Nup	nucleoporin
O.D.	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pH	$-\log[H^+]$
poly(A)+	polyadenylated RNA
psl	Pom152 synthetic lethal
RNA	ribonucleic acid
RNP	ribonucleoprotein
SDS	sodium dodecyl sulphate
$Sec^{+/-}$	sectoring/non-sectoring colony

snRNP	small nuclear RNP
TCA	trichloroacetic acid
ts	temperature sensitive
U	units of enzyme
UV	ultraviolet light
V	Volts
v	volume
w	weight
Ω	ohm
ψ	pounds per square inch

Chapter 1

Introduction

1.1 The Nuclear Envelope

The defining structural feature of eukaryotic cells is the nucleus. The nucleus is delimited by the nuclear envelope (NE), a double membrane bilayer that acts as a selectively permeable barrier between the nucleoplasm and the cytoplasm and offers a means of regulating gene expression that is unavailable to prokaryotes. The nuclear envelope is composed of three biochemically distinct membrane domains: the outer nuclear membrane, the inner nuclear membrane, and the pore membrane domain (see *Figure 1-1*; Akey and Radermacher, 1993; reviewed in Goldberg and Allen, 1995). The outer nuclear membrane interfaces with the cytoplasm, is continuous with the endoplasmic reticulum, and is decorated with ribosomes. The inner nuclear membrane lines the inner aspect of the nucleus and contains a unique set of proteins. The inner nuclear membrane of vertebrate cells, but not yeast, is lined with a network of lamin filaments forming an array that is thought to maintain the structure of the NE. The inner nuclear membrane and outer nuclear membrane join at multiple sites forming aqueous channels, or pores, through which the nucleoplasm and the cytoplasm are connected. The pore membrane domain lining these pores is occupied by integral, and peripherally associated membrane proteins that form macromolecular structures, called nuclear pore complexes (NPCs), which serve to regulate nucleo-cytoplasmic exchange.

1.2 Structure of the Nuclear Pore Complex

The basic structural framework of the vertebrate NPC has been defined from amphibian oocytes using electron microscopy (Akey and Radermacher, 1993; Goldberg et al., 1995; reviewed in Rout and Wente 1994; Goldberg and Allen, 1995). These studies have provided a low resolution model of the vertebrate NPC. The NPC consists of a cylindrical core composed of eight equally spaced spokes, which are connected to adjacent neighbors by four coaxial rings (see *Figure 1-1*). The cytoplasmic ring and nucleoplasmic ring are coplanar to the outer nuclear membrane and inner nuclear membrane respectively, the outer spoke ring is in the lumen of the NE, and the inner spoke ring surrounds a central plug (Akey and Radermacher, 1993). The central plug or "transporter" is positioned in the center of the spoke-ring complex and occupies most of the space delineated by the NE. A significant portion of the spokes appears to extend across the pore-membrane domain into the lumen of the NE. The spoke-ring complex and central transporter together form the NPC core structure, which has been measured in amphibian oocytes at approximately 120 nm in diameter and 37 nm in height (Akey and Radermacher, 1993). Emanating from the highly symmetrical spoke-ring complex are two morphologically distinct and peripherally associated structures. Eight short filaments extend approximately 100 nm into the cytoplasm and attach to the cytoplasmic ring. In contrast, eight filaments projecting into the nucleoplasm are joined by a terminal ring forming a basket (50-100 nm deep). In addition, filaments originating from deep within the nucleus are attached to the distal ring of the nuclear basket, and may form tracks for the movement of molecules, suggesting that the influence of the NPC extends far beyond the region proximal to the NE (Meier and Blobel 1992; Zimowska et al., 1997; Cordes et al., 1997; Shah et al., 1998; Kosova et al., 2000).

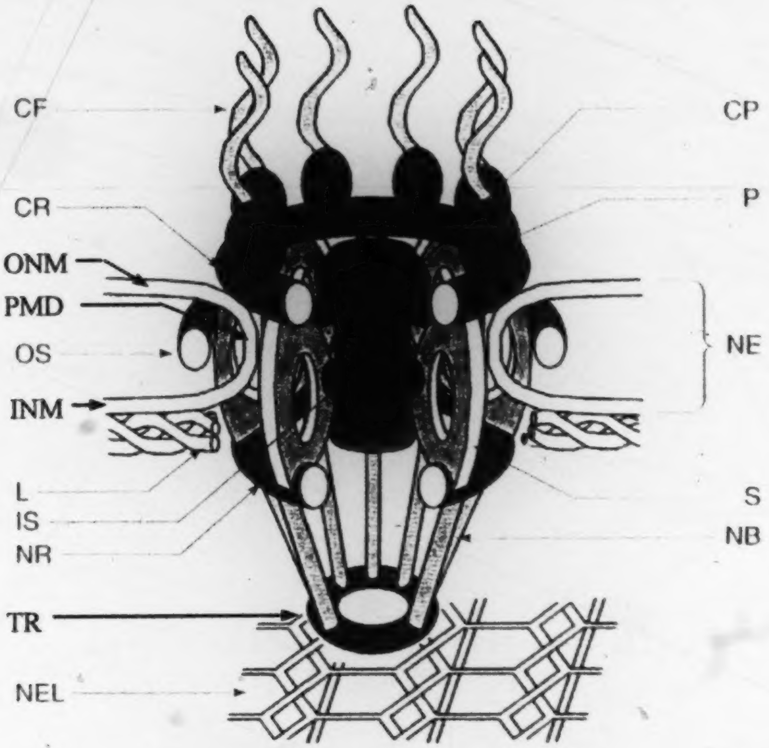


Figure 1-1. The nuclear pore complex. Three dimensional model of the vertebrate NPC is based on data obtained from scanning and cryo-electron microscopy (Akey and Radermacher, 1993). The model illustrates the basic structural features of the vertebrate NPC, drawn approximately to scale. The cytoplasmic face is uppermost and some of the facing features have been deleted in order to view the transporter, or central plug (CP). Other features include the cytoplasmic filaments (CF); cytoplasmic rings (CR), outer spoke ring (OS), inner spoke ring (IS), nuclear ring (NR), cytoplasmic particle (P); outer nuclear membrane (ONM); pore membrane domain (PMD); inner nuclear membrane (INM); spoke (S); nuclear basket (NB); terminal ring (TR); lamina (L); and nuclear envelope lattice (NEL). The vertebrate NPC has been calculated to be 120 nm in diameter and 70 nm in length (perpendicular to the plane of the NE). Model adapted from Rout and Wente (1994).

1.3 Conservation of Nuclear Pore Complex Structure

It has been established that the vertebrate NPC is made up of approximately 50 different proteins (Fontoura et al., 1999), termed nucleoporins, that contribute to the 125 megadaltons (MD) estimated mass of the vertebrate NPC (Reichelt et al., 1990). In contrast, the yeast NPC is composed of about 30 nucleoporins with a combined mass of 50 MD (Rout and Blobel, 1993; Rout et al., 2000), but it conserves the same basic function and overall design of its vertebrate counterpart (see *Figure 1-2*) (Yang et al., 1998). The mass difference between the vertebrate and yeast NPC is largely due to the lack of structural features such as the inner spoke ring, and the peripheral cytoplasmic and nuclear rings. The absence of the abundant transmembrane proteins homologous to the vertebrate gp210p and Pom121p may also contribute to a smaller lumenal spoke mass of the yeast NPC (Yang et al., 1998). However, the conservation of yeast and vertebrate NPCs is not limited to structure alone. Tremendous advances in the identification of nucleoporins from yeast to humans have allowed the identification of homologous nucleoporins across species (see *Table 1-2*; reviewed in Stoffler et al., 1999). For example, several vertebrate nucleoporins have been shown to functionally complement yeast mutants (Aitchison et al., 1995a; Strahm et al., 1999). Taken together, the conservation of overall structure and the functional homology of several NPC components suggests that the NPCs of yeast and vertebrates have common evolutionary origins.

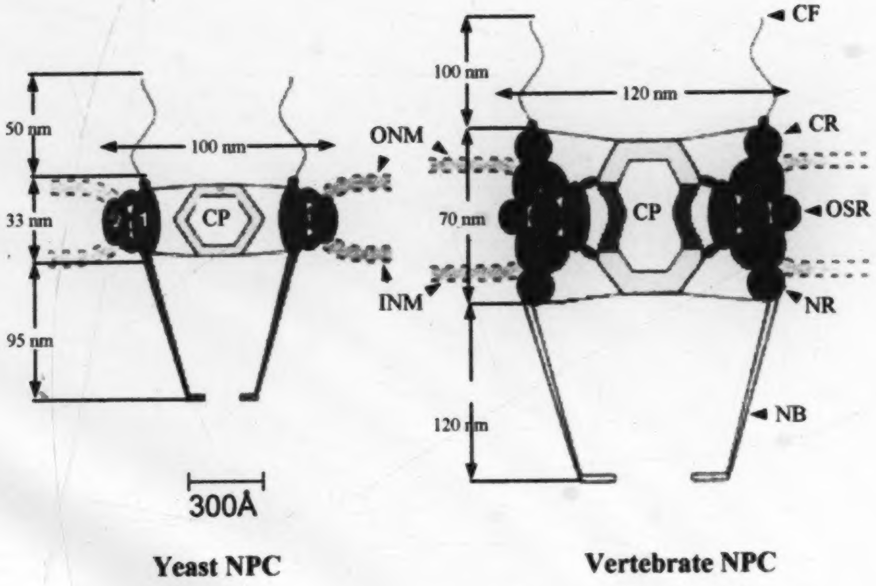

50 nm
100 nm
33 nm
95 nm
CP
300Å
Yeast NPC
ONM
INM
CF
100 nm
120 nm
CR
70 nm
CP
OSR
NR
NB
120 nm
Vertebrate NPC

Figure 1-2. Comparison of yeast and vertebrate NPCs. Idealized models of a yeast (left) and vertebrate (right) NPC are shown in cross section. The structural components of the vertebrate NPC are: cytoplasmic filaments (CF), cytoplasmic ring (CR), outer spoke ring (OSR), nuclear ring (NR), nuclear basket (NB), and central plug (CP). The yeast NPC is less massive and is composed of two radial spoke domains (1 and 2), and central plug (CP). Putative nuclear basket and cytoplasmic filaments are shown on the yeast NPC. Conserved structural components of the NPC core appear in matching colors. The dimensions of various structural features are indicted on both models for comparison. The thickness of the NE lumen appears to correspond to the vertical dimensions of the NPC core. Figure modified from Akey and Radermacher (1993) and Yang et al. (1998).

1.4 Nucleo-Cytoplasmic Transport

Although the NPC has been speculated to play a role in the organization of the nucleoplasm, the regulation of gene expression, and the structural organization of the nucleus (reviewed in Blobel, 1985; and Dessev, 1992). However, the most studied and well characterized function of the NPC is in the regulation of bi-directional transport of macromolecules into and out of the nucleus. The structure of the NPC contains aqueous channels of approximately 10 nm in diameter, which can account for the passive diffusion of ions, metabolites, and possibly polypeptides of less than 50 kilodaltons (kD) (Paine et al., 1975). However, the translocation of most polypeptides, ribonuceloproteins (RNPs) and small nuclear RNPs is saturable (Zasloff et al., 1983; Goldfarb et al., 1986), energy-dependent (Zasloff et al., 1983; Newmeyer et al., 1986), and signal-mediated (Dingwall and Laskey, 1991). This process can accommodate the translocation of macromolecules as large as 30 nm in diameter, and corresponding to millions of Daltons, through the NPC (Feldherr et al., 1984; reviewed in Talcott and Moore, 1999; Mattaj and Engelmeier, 1998).

1.5 Signal-Mediated Transport

The translocation of most proteins through the NPC is dependent on cis-acting signal sequences. Proteins containing a nuclear localization signal (NLS) are imported into the nucleus, whereas those containing a nuclear export signal (NES) are exported from the nucleus (Gerace et al., 1995; Fischer et al., 1996). There is no consensus for either NLSs or NESs, but they are generally short peptides that may occur anywhere within the sequence of the protein, and are not cleaved upon transport. NLSs and NESs share the common property of promoting the translocation of heterologous reporter proteins into (NLSs) or out of (NESs) the nucleus. Amongst these, perhaps the most common and most studied NLS is known as the "classical" NLS (cNLS). Although there is no clear consensus sequence for the cNLSs, they are frequently composed of short sequences of basic amino-acid residues (Dingwall and Laskey, 1991). These signals are recognized by soluble receptors, or karyopherins (from the Greeks words for nucleus "karnon" and "pherein" meaning to bring to, or to carry from (Radu et al., 1995a); also known as importins, transportins, or exportins), that mediate the translocation of their cargo through the NPC.

1.6 Karyopherin α/β-Mediated Nuclear Import

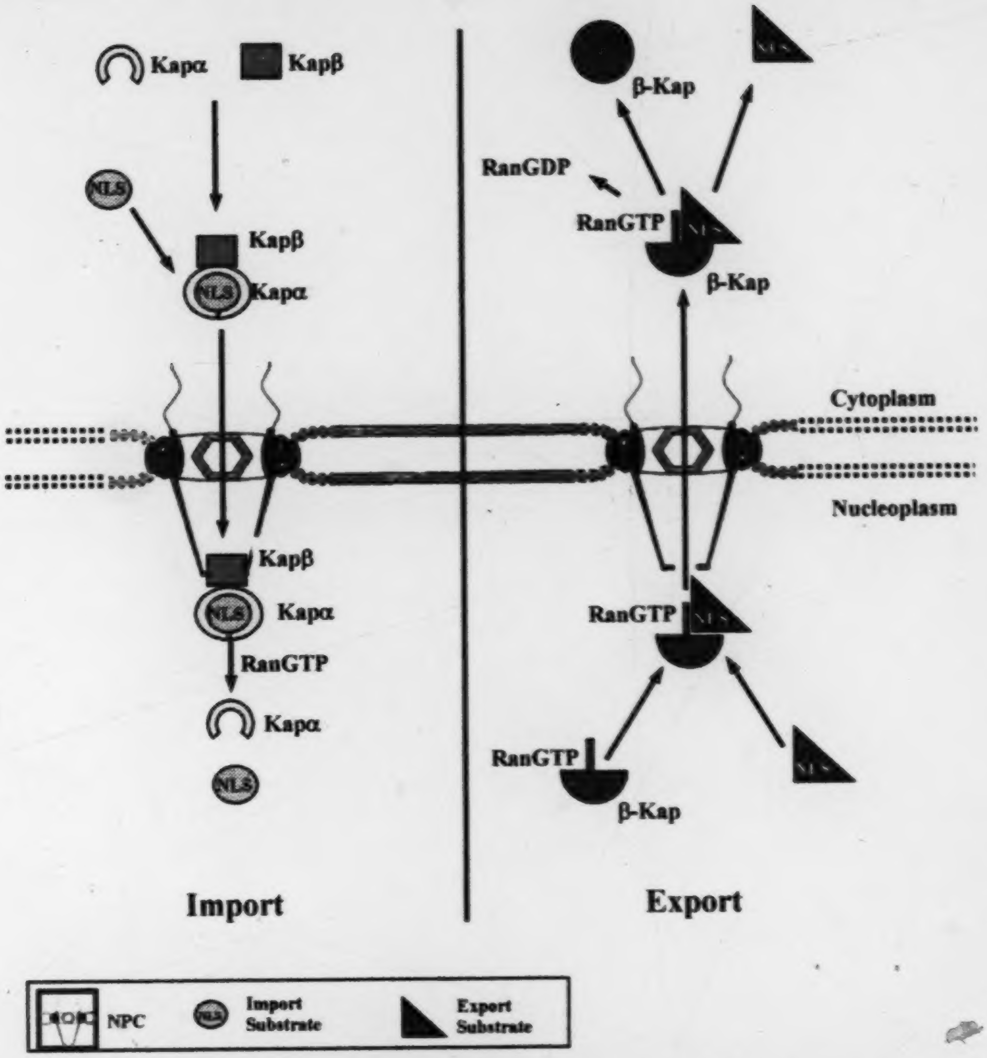
Much of what is currently known about signal-mediated nuclear import originates from studies examining the import of molecules containing a basic cNLS similar to that present in the large T antigen of Simian Virus 40 (PKKKRKV). These studies determined that the import process could be divided into two distinct steps: docking of the cargo to the NPC and energy-dependent translocation (reviewed in Görlich and Mattaj, 1996). The development of an *in vitro* nuclear import assay, using semi-permeabilized tissue culture cells (Adam et al., 1990), led to the identification and purification of soluble factors necessary for import. These included karyopherin α (Kap α) (Radu et al., 1995; also referred to as the NLS receptor [Adam and Gerace, 1994; Adam and Adam, 1994; Imamoto et al., 1995a] or importin α [Görlich et al., 1994]), karyopherin β (Kap β) (Radu et al., 1995; also known as importin β [Görlich et al., 1995] and p97 [Chi et al., 1995; Imamoto et al., 1995a]), the small GTPase Ran (Moore and

Blobel, 1993; Melchior et al., 1993), and NTF2 (also known as p10 [Moore and Blobel, 1994; Paschal and Gerace, 1995; Nehrbass and Blobel, 1996; Ribbeck et al., 1998; Smith et al., 1998]).

The role that these factors play in transport has been defined experimentally and several stages in the transport of cNLS-bearing proteins have been established (see *Figure 1-3*). Import substrates containing a cNLS are recognized and bound by an NLS receptor heterodimer comprised of Kap α (Kap60p in yeast) and Kap β (Kap95p in yeast). Kap α has been shown to provide the cNLS binding site (Adam and Gerace 1991; Görlich et al., 1994; Weiss et al., 1995; Imamoto et al., 1995b), whereas Kap β serves to dock the cNLS/Kap α/Kap β complex at the NPC (Moroianu et al., 1995; Görlich et al., 1995b). Electron microscopy studies using gold particles coated with the protein nucleoplasmin suggested that the cNLS/Kap α/Kap β complex docks at multiple sites on the cytoplasmic filaments of the NPC (Feldherr et al., 1984; Richardson et al., 1988; Akey and Goldfarb, 1989; Pante and Aebi, 1996). The import complex subsequently moves from the filaments to the cytoplasmic face of the NPC core by what has been proposed to be a series of docking and release steps (Radu et al., 1995b). The receptor-cargo complex then appears to translocate through the transporter in the center of the NPC. The cNLS/Kap α/Kap β complex may then associate with the nucleoplasmic filaments and the cNLS-containing polypeptide and Kap α are released into the nucleoplasm (Moroianu et al., 1995; Görlich et al., 1995b). The detection of Kap β at both sides of the NPC by immunoelectron microscopy suggests that Kap β translocates with cNLS/Kap α into the nucleoplasmic face of the NPC (Görlich et al., 1995b). However, unlike Kap α, Kap β does not accumulate in the nucleoplasm, suggesting that it is rapidly exported to the cytoplasm for another round of import. Kap α is also ultimately recycled to the cytoplasm, but at a different rate, and by a separate mechanism (Kutay et al., 1997; see below).

1.7 The Role of Ran in Nuclear Transport

After the cNLS/Kap α/Kap β complex has docked to the NPC, the subsequent translocation through the NPC is energy-dependent. The energy requirements for translocation are not well understood, but it depends in part on the function of the small GTPase Ran (reviewed in Cole and Hammell, 1998; Melchior and Gerace, 1998; Mattaj and Engelmeier, 1998). Ran is predominantly found in the nucleoplasm, but its presence in the cytoplasm suggests that it might shuttle across the nuclear envelope. Ran exists mainly in two forms, either bound to GTP or to GDP. The nucleotide-bound state is maintained by two Ran regulator molecules, a Ran GTPase activating protein RanGAP (Rna1p in *S. cerevisiae*) (Atkinson et al., 1985; Bischoff et al., 1995) and a Ran guanine nucleotide exchange factor, RCC1 (Prp20p in *S. cerevisiae*) (Bischoff and Ponstingl, 1991; Clark et al., 1991). In the presence of RanGAP, Ran is stimulated to hydrolyze GTP (Bischoff et al., 1995). By contrast, RCC1 stimulates the production of RanGTP (Bischoff and Ponstingl, 1991). A characteristic feature of the Ran system is the differential distribution of its constituents. RanGAP is excluded from the nucleus and depletes RanGTP from the cytoplasm (Hopper et al., 1990; Richards et al., 1996; Mahajan et al., 1997; Matunis et al., 1997), but RCC1 is chromatin-bound and generates RanGTP in the nucleus (Ohtsubo et al., 1989). The asymmetrical localization of the Ran effector proteins on opposite sides of the NE suggests that Ran is predominantly GDP


Kapα
Kapβ
NLS
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NLS
Kapα
Kapβ
NLS
Kapα
RanGTP
Kapα
NLS
β-Kap
RanGDP
RanGTP
β-Kap
Cytoplasm
Nucleoplasm
RanGTP
RanGTP
β-Kap
Import
Export
NPC
Import Substrate
Export Substrate

Figure 1-3 β-karyopherin-mediated nuclear transport. **Nuclear Import:** The general features of β-karyopherin-mediated import are outlined using the Kap α/Kap β import system as an example. A complex of Kapα, Kapβ and the import substrate (NLS) is formed in the cytoplasm where RanGTP levels are low. The complex docks to the NPC via Kapβ and is translocated into the nucleus. Nuclear RanGTP causes the dissociation of the import complex releasing Kapα and the NLS into the nucleus. Kapβ is rapidly recycled for another round of import. Kapα is also recycled to the cytoplasm by karyopherin CAS. **β-karyopherin-Mediated Export.** An export receptor (β-Kap) forms a complex with RanGTP and the export substrate (NES). This complex is exported from the nucleus and is dissociated in the cytoplasm by the hydrolysis of RanGTP to RanGDP stimulated by a Ran GAP present in the cytoplasmic face of the NPC and in the cytoplasm. The export factor is recycled to the nucleus for another round of import.

bound in the cytosol and GTP bound in the nucleus (Görlich et al., 1996a; Görlich et al., 1996b). The concentration gradient of RanGTP and RanGDP across the NE has been proposed to play a key role by imparting directionality on nucleocytoplasmic transport (see Cole and Hammell, 1998). For example, the Kap α/Kap β complex was shown to bind a cNLS-containing polypeptide in the presence of Ran or RanGDP, a condition that may occur in the cytoplasm (Rexach and Blobel, 1995). However, both pre-formed cNLS/Kap α/Kap β complexes or Kap α/Kap β bound to a nucleoporin were readily dissociated by the binding of RanGTP to Kap β (Rexach and Blobel, 1995; Percipalle et al., 1997). These results and others suggested that RanGTP may drive the binding and release steps through the NPC that occur in the import process (Rexach and Blobel, 1995) and in the terminal dissociation of the import complex at the nucleoplasmic face of the NPC (Görlich et al., 1996a). Thus, Ran imparts directionality by favoring the formation of import complexes in the cytoplasm and stimulating their dissociation in the nucleoplasm (and *vice versa* for export). However, the hydrolysis of GTP by Ran is not necessary for (at least one round of) transport (Richards et al., 1997; Schwoebel et al., 1998; Ribbeck et al., 1999; Englmeier et al., 1999).

1.8 Other Transport Pathways

The basic cNLS is only one of a growing number of NLSs and NESs. A number of import and export substrates have recently been identified that contain unique signal sequences that are recognized by different receptors. The vast majority of receptors share limited sequence homology to each other and to Kap β, and are collectively known as the β-karyopherins. In yeast, fourteen potential β-karyopherins have been identified by primary amino-acid sequence similarity (reviewed in Wozniak et al., 1998) and the number in humans is likely to be more than twenty (Pemberton et al., 1998). Individual members of the β-karyopherins have been shown, in both yeast and mammalian cells, to transport specific classes of cargo through the NPC (*Table 1-1*). For example, in yeast, Kap123p/Kap β4 has been shown to mediate the nuclear import of the ribosomal protein rpL25 (Rout et al., 1997; Yaseen and Blobel, 1997; Schlendstedt et al., 1997), and Kap121p/Kap β3 the import of the transcription factor Pho4p (Kaffman et al., 1998a).

The export of nuclear proteins and ribonucleoproteins (RNPs) is also an active process requiring specific members of the β-karyopherin family (see *Table 1-1*; reviewed in Nakielny and Dreyfuss, 1999). However, unlike import, the interaction between a β-karyopherin and its cognate export substrate appears to occur with much higher affinity in the presence of RanGTP (Fornerod et al., 1997a; Fukuda et al., 1997; Kutay et al., 1997; Arts et al., 1998). A trimeric complex composed of the β-karyopherin, its cargo, and RanGTP is translocated through the NPC into the cytoplasm (see *Figure 1-3*), and RanGAP activity at the cytoplasmic face of the NPC and its coactivator RanBP1 stimulate the disassembly of the export complex and the release of the cargo into the cytoplasm (Kutay 1997; Matunis et al., 1998; Mahajan et al., 1998; Kehlenbach et al., 1999). Several β-karyopherins that mediate export and their cognate cargo have been identified. Amongst these, Crm1p/exportin has been shown, in both yeast and vertebrates, to mediate the export of proteins containing a 10-amino-acid residue, leucine-rich NES like that found within the HIV-Rev protein (see *Table1-1*; Fornerod et al., 1997a; Fukuda et al., 1997; Neville et al., 1997; Ossareh-Nazari et al., 1997; Stade et al., 1997). Cse1p in yeast and CAS in vertebrates have been shown to mediate the recycling of Kap α (Kutay et al., 1997; Herold et al., 1998; Hood and Silver, 1998; Kunzler and Hurt, 1998; Solsbacher et al., 1998). Likewise the export of tRNA is mediated by Los1/exportin-t (Arts et al., 1998; Kutay et al., 1998; Sarkar and Hopper, 1998) and in higher eukaryotes the export of U snRNA and associated proteins appear to be mediated by Crm1 (Jarmolowski et al., 1994; Izaurralde et al., 1995; Fornerod et al., 1997a) However, despite the specifity of individual karyopherins for their cargo proteins, some functional overlap between β-karyopherins has been reported. For example, the import of the ribosomal protein rpL25p can be facilitated by Kap121p in the absence of Kap123p (Rout et al., 1997; Schlenstedt et al., 1997). Likewise, *KAP121* deletions, which result in a lethal phenotype, can be functionally complemented by an additional copy of a gene encoding a different β-karyopherin *SXM1* (Seedorf and Silver, 1997).

1.9 Regulation of β-karyopherin-Mediated Transport

The multiple steps and factors involved in the translocation of a substrate through the NE provide a means of potentially modulating transport by regulating karyopherin-substrate recognition, docking at the NPC, or translocation through the NPC. The identification of individual karyopherins and their cargo proteins is only the first step in elucidating some of these possible control mechanisms (reviewed in Hood and Silver, 1999). Recent studies have shown that reversible phosphorylation of specific substrates in response to stress (Kuge et al., 1997; Ferrigno et al., 1998; Gaits et al., 1998; Toone et al., 1998), or nutrient starvation (Kaffman et al., 1998a; Kaffman et al., 1998b; DeVit et al., 1997), provide a means of modulating the affinity of the substrate for their cognate karyopherins. One example involves the differential localization of the transcription

Table 1-1 Yeast β-Karyopherins

Name	Class	Homologues	Transport	Cargo	Cargo function
Kap95p	Kap β1	Karyopherinβ1 Importin β	Import	proteins containing a cNLS	Wide-ranging
Kap104p	Kap β2	Transportin 1 Transportin 2	Import	hnRNPA1, A2, F Nab2p, Nab4p/Hrp1p	mRNA export
Kap108/Sxm1p	-	RanBP7 RanBP8	Import	Lph1p	Factor for tRNA maturation
Kap111/Mtr10p	-	-	Import	Npl3p	mRNA binding protein
Kap114p	-	-	Import	TBP	Transcription
Kap119/Nmd5p	-	RanBP7 RanBP8	Import	Hog1 TFIIS	MAP Kinase RNA pol II transcription elongation factor
Kap121p/Pse1p	Kap β3	PSE1	Import	ribosomal proteins	Translation
Kap122p/Pdr6p	-	-	Import	TFIIA	Transcription factor
Kap123p/Yrb4p	Kap β4	-	Import	ribosomal proteins	Translation
Crm1	-	exportin 1	Export	leucine rich NES	Wide-ranging
Los1	-	exportin t	Export	tRNA	Translation
Cse1p	-	CAS	Export	Karyopherinα	Kap60p/Kapα
Msn5p	-	-	Export	Pho4p Far1p	Transcription factor Regulates cell cycle/cytoskeleton
YPL125W	-	-	-	-	-

S.cerevisiae β-karyopherins listed with alternate names. Transport refers to whether a karyopherin has been shown to be involved in the import or export of its cognate cargo. See text for references (or Wozniak et al., 1998; Nakielny and Dreyfuss, 1999).

factor Pho4p in yeast cells. Its import into the nucleus is regulated by its phosphorylation state. In its phosphorylated form Pho4p is cytoplasmic. When dephosphorylated, in response to decreased levels of free phosphate ions, Pho4p binds to Kap121p and is imported into the nucleus (Kaffman et al., 1998a). Conversely, in response to an increase in phosphate ions, Pho4p is phosphorylated and its export from the nucleus is mediated by Msn5p, which recognizes only the phosphorylated form of Pho4p (Kaffman et al., 1998b). The modulation of substrate affinity for specific karyopherins results in an exquisitely controlled regulation of karyopherin-dependent translocation. Phosphorylation may also play a role in altering the interaction between nucleoporins and karyopherins. The regulation of transport by phosphorylation at the level of the NPC has not been identified. As discussed in Chapter 3, the mitotic phosphorylation of a yeast nucleoporin that acts as binding site for a β-karyopherin may modulate transport at specific stage of the cell cycle.

1.10 The Nucleoporins

Despite the diversity of β-karyopherins and their associated cargos, the NPCs provide the sole sites for their nucleo-cytoplasmic exchange. This translocation occurs by what has been proposed to be a series of binding and release reactions within the NPC; however, the exact mechanism behind this translocation remains elusive. In an effort to elucidate this mechanism, much attention has been focused on the identification of nucleoporins and NPC-associated proteins and the characterization of their role in the transport process. Biochemical and genetic approaches have led to the identification of numerous nucleoporins and NPC-associated components. Amongst these, many nucleoporins have been isolated in both yeast and vertebrates on the basis of their reactivity to polyspecific monoclonal antibodies (Davis and Blobel, 1986; Davis and Blobel, 1987; Snow et al., 1987; Wente et al., 1992), their presence in enriched fractions of NPCs, and in affinity-purified NPC subcomplexes (reviewed in Doye and Hurt 1997). In addition, many yeast nucleoporins were found as a result of genetic screens to identify interactions with known nucleoporins (synthetic lethal screens) and by screens used to isolate mutants in both poly(A)$^+$ RNA export and nuclear protein localization (Amberg et al., 1992; Kadowaki et al., 1992; Bossie et al., 1992; reviewed in Doye and Hurt, 1997).

The classical approaches aimed at identifying nucleoporins have been extensive and fruitful and it now appears that the search for new nucleoporins in yeast is nearing an end. Recently mass spectrometry approaches have been used to identify the vast majority of polypeptides in a fraction of highly enriched yeast NPCs (Rout et al., 2000). The subsequent characterization of these proteins revealed two new nucleoporins bringing the total number up to ~30 (see *Table 1-2*). Interestingly, most of these components had been previously identified as nucleoporins by genetic approaches and biochemical analyses. In contrast, to date fifteen out of an estimated fifty vertebrate genes encoding nucleoporins have been identified (reviewed in Stoffler et al., 1999; Rout and Wente, 1994). However, amino-acid sequence similarity between yeast and vertebrate NPC components will likely expedite the identification of new vertebrate homologues concomitant with progress in the sequencing of the higher eukaryotic genomes.

The NPC is a dynamic structure with which a number of proteins transiently associate, like karyopherins and their cargo, as they translocate through the NPC. On the

Table 1-2 Saccharomyces cerevisiae Nucleoporins

Name(s)	Homologues	FG Repeats[a]	Disruption[b]	Mutants defective in	Localization[c]
Nup1 (113 kDa)	-	FXFG	Lethal	-	N
Nsp1 (86 kDa)	*r,h,x* p62	FXFG	Lethal	Import NPC/NE	C/N
Seh1p (39 kDa)	*m,r* Sec13	-	Viable	-	-
Ndc1p (74 kDa)	-	-	Lethal	Spindle pole	Transmembrane[d]
Gle1p/Rss1 (62 kDa)	-	-	Lethal	Export	C/n
Gle2p (41 kDa)	*Sp*Rae1p	-	Viable	Export NPC/NE	C/N
Nup2 (78 kDa)	-	FXFG	Viable	Export	C/N
Sec13p (32 kDa)	*m,r* Sec13	-	Lethal	-	-
Pom34p	-	-	Viable	-	Transmembrane[d]
Nup42p/Rip1P	*h*RIP/Rab	FG	Viable	Import Export	C
Nup49p	*r* p58 *Sp*Nup49p	GLFG	Lethal	Import Export	C/N
Nup53p	*x*MP44	FG	Viable	Import	C/N
Nup57p	*r* p54/p45 *Sp*Nup57p	GLFG	Lethal	Import, export	C/N
Nup59p	*x*MP44	FG	Viable	Import	C/N
Nup60p	-	FXFG	Viable	-	N
Nup82p	-	-	Lethal	Import, export	C
Nup84p	*r*Nup107	-	Viable	Export NPC/NE	C/N
Nup85p/Rat9p	-	-	Viable	Export NPC/NE	C/N
Nic96p	*h,r,x*Nup93p	-	Lethal	Import NPC/NE	C/N
Nup100p	-	GLFG	Viable	-	C/n
Nup116p/Nsp116p	*h,r,x*Nup98	GLFG	Viable	Export NPC/NE	C/n
Nup120p/Rat2p	-	-	Viable	Export NPC/NE	C/N
Nup133p/Rat3p	-	-	Viable	Export NPC/NE	C/N
Nup145p/Rat10p	C-ter *h*Nup96	GLFG	Lethal	Export NPC/NE	C-ter C/N N-ter c/N
Pom152p	-	-	Viable	-	Transmembrane[d]
Nup157p	*r*Nup155 *dm*Nup154	-	Viable	-	C/N
Nup159p/Rat7p	-	FG	Lethal	Export NPC/NE	C
Nup170p	*r*Nup155 *dm*Nup154	-	Viable	NPC/NE	C/N
Nup188p	-	-	Viable	NPC/NE	C/N
Nup192p	*r,h* p205	-	-	-	C/N

Yeast nucleoporins are listed in ascending order of their designated name, according to their predicted molecular mass or genetic identification. The designations Nup (nuclear pore protein) and Pom (for integral membrane protein of the pore membrane) are used for both yeast and vertebrate polypeptides. Lower case letters indicate species: *r*, rat; *h*, human; *m*, mouse; *x*, *Xenopus laevis*; *Sp*, *Schizosaccharomyces pombe*; *dm*, *Drosophila melonagaster*. C-ter and N-ter refers to the N-terminus or C-terminus of Nup145p. [a] Indicates nucleoporins containing FXFG, GLFG, or degenerate FG repeats. [b] indicates whether null mutants of the indicated nucleoporin gene results in a viable or lethal phenotype. [c] Localization determined by immunoelectron microscopy analyses: C/N, both faces of the NPC; C/n, showed a bias towards the cytoplasmic aspect of the NPC; c/N, showed a bias towards the nucleoplasmic aspect of the NPC; C, exclusively on the cytoplasmic face of the NPC; N, exclusively on the nucleoplasmic face of the NPC. [d] Integral membrane proteins of the pore membrane. For references see text or Doye and Hurt (1997); Stoffler et al. (1999), and Rout et al., 2000.

other hand, the *bone fide* nucleoporins represent the proteins that are stably associated with, and spend the majority of their time as components of the NPC. The known nucleoporins, in both yeast and vertebrates, can be roughly subdivided into two groups: The first, consists of a family of nucleoporins referred to as the FG repeat-containing nucleoporins that contain degenerate FXFG, GLFG, or FG repeats (single amino-acid code where X represents a small residue) and represents roughly one-half of the yeast nucleoporins and the majority of the known vertebrate nucleoporins. The second group contains those nucleoporins lacking this motif. Here, I will discuss the nucleoporins of the yeast *Saccharomyces cerevisiae* with specific reference to mammalian nucleoporins where appropriate.

1.11 The FG Repeat-Containing Nucleoporins Are Involved in Transport

On the basis of genetic and biochemical analyses the FG repeat-containing family of nucleoporins has been suggested to play a direct role in transport. The evidence for this includes the observation that several FG repeat-containing nucleoporins have been shown to interact with β-karyopherins by ligand blot overlay assays, *in vitro* binding assays, and two hybrid analyses (Dingwall et al., 1995; Kraemer et al., 1995; Nehrbass and Blobel, 1996; Aitchison et al., 1996; Fornerod et al., 1997b; Iovine and Wente, 1997; Yaseen and Blobel, 1997; Morioianu and Blobel, 1997; Shah and Forbes, 1998a; Shah et al., 1998; Bailer et al., 1998; Boothe et al., 1999; Seedorf et al., 1999; Nakielny et al., 1999; Uetz et al., 2000; see also Chapter 3). Functionally, mutations in several FG repeat-containing proteins have been shown *in vivo*, in both yeast and vertebrates, to have profound effects on import, export or both (reviewed in Stoffler et al., 1999). For example in yeast, mutations of *NUP116*, *NUP1*, *NSP1*, and several other genes encoding FG repeat-containing nucleoporins, display defects in the transport of nuclear proteins or RNA (see *Table 1-2*). Likewise, the role of the FG repeat-containing nucleoporins in transport has been investigated in higher eukaryotes. Both monoclonal and polyclonal antibodies specific for the FG repeat-containing nucleoporin p62 blocked import in *Xenopus* oocytes (Featherstone et al., 1988, Debauvalle et al., 1988). In cultured mammalian cells, overexpression of the vertebrate *NUP153* (encoding Nup153p, an FG repeat-containing nucleoporin of the nuclear basket) was used successfully to define the role of this protein in mRNA export (Bastos et al., 1996). Deletions of the gene encoding CAN/Nup214p in murine embryos resulted in a lethal phenotype that was associated with defects in both nuclear protein import and mRNA export (Van Deursen et al., 1996). These results indicate that the FG repeat-containing nucleoporins have a functional role in the translocation process.

The FG repeat-containing nucleoporins have been localized to substructures of yeast and vertebrates NPCs based on immunoelectron microscopy techniques (see *Figure 1-4*). For example, yeast Nup159p and Nup42p (Kraemer et al., 1997; Rout et al., 2000), and vertebrate CAN/Nup214p, and Nup358p (Kraemer et al., 1994; Fornerod et al., 1995; Wu et al., 1995; Yokoyama et al., 1995) are predicted to be components of the cytoplasmic filaments due to their exclusive localization to the cytoplasmic side of the NPC. Likewise, the localization of the yeast Nup60p and Nup1p (Fahrenkrog et al., 1998; Rout et al., 2000), and vertebrate Nup98p, Tpr, and Nup153p (Sukegawa et al., 1993; Powers et al., 1995; Radu et al., 1995b; Zimowska et al., 1997; Cordes et al., 1997; Bangs et al., 1998) solely to the nuclear side of the NPCs suggests that they are

Figure 1-4 Substructural Location of Yeast and Vertebrate Nucleoporins. Several nucleoporin subcomplexes have been identified from yeast and vertebrate cells by immunoprecipitation and co-fractionation (Doye and Hurt, 1997). Immunoelectron microscopy analyses of individual subcomplex components have localized most nucleoporins to both sides of the NPC. These, along with their associated subcomplex(es), are thought to be components of the repetitive structures of the NPC core. A handful of nucleoporins are distributed to either the nuclear or cytoplasmic aspect of their associated NPCs. Due to their biased localization, these are thought to compose the asymmetrical structures of the NPC, namely the nuclear basket, or the cytoplasmic filaments respectively. However, how the localization of these components relates to substructures of the NPC remains to be determined. Tremendous advances in the identification of nucleoporins across species have allowed sequence comparison and the identification of homologous pairs (see Stoffler et al., 1999). To date, homologues to two thirds of the yeast nucleoporins have been identified in vertebrates (see *Table 1-2*). In addition, the components of many subcomplexes appear to be conserved across species (Carmo-Fonseca et al., 1991 Radu et al., 1994; Radu et al., 1995; Powers et al., 1995; Aitchison et al., 1995a; Kenna et al., 1996; Sinissoglou et al., 1996 Grandi et al., 1997). Yeast and vertebrate homologues and conserved subcomplexes have been listed across from each other wherever possible..

FG repeat-containing nucleoporins are listed in green, and integral pore-membrane proteins in red. A schematic representation of the yeast and vertebrate NPC is shown under their appropriate column in relief.

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1. Gorsch et al. 1995; Kraemer et al., **1995.** **2.** Rout et al., 2000. **3.** Marelli et al., 1998. **4.** Wimmer et al., 1992; Grandi et al., 1993; Fahrenkrog et al., 1999; Carmo-Fonseca et al., 1991. **5.** Grandi et al., 1995; Fahrenkrog et al., 1999. **6.** Nehrbass et al., 1996; Zabel et al., 1996. **7.** Rout et al., 2000 **8.** Chial et al., 1998. **9.** Sinissioglou et al., 1996; Radu et al., 1994; Sinissoglou et al., 1996. **10.** Kosova et al., 1999. **11.** Wu et al., 1995. **12.** Kraemer et al., 1994; Fornerod et al., 1996. **13.** Wilken et al. 1993. **14.** Radu et al., 1993. **15.** Finlay et al., 1991; Guan et al., 1995; Hu et al., 1996; Schlaich et al., 1996; Carmo-Fonseca et al., 1991. **16.** Hallberg et al., 1993. **17.** Greber et al., 1990; Wozniak et al., 1992. **18.** Grandi et al., 1995; Grandi et al., 1997. **19.** Radu et al., 1994; Grandi et al., 1997; Radu et al., 1994; Sinissoglou et al., 1996. **20.** Radu et al., 1995 **21.** Sukegawa et al., 1993; Cordes et al., 1993. **22.** Zimowska et al., 1997; Cordes et al., 1998; Bangs et al., 1998.

	Yeast	Vertebrates
Cytoplasmic	Nup159p[1] Nup42p[2]	Nup358p[11] CAN/Nup214p-Nup88p[12] Nup180p[13]
NPC Core	Nup157p[3] Nup53p-Nup170p-Nup59p[3] Nsp1p-Nup49p-Nup57p-Nic96p[4] Nsp1p-Nup82p[5] Nup188p-Nic96p-Pom152p[6] Pom34p[7] Ndc1p[8] Nup84p-Nup85p-Nup120p[9] Nup145C-Seh1p	Nup155p[14] p62-p58-p54-p45[15] Pom121p[16] gp210[17]
Nucleoplasmic	Nup192p-Nic96p[10] Nup1p[7] Nup60p[7]	Nup93p-p205p[18] Nup96p-Nup107p[19] Nup98p[20] Nup153p[21] Tpr[22]

components of the nucleoplasmic basket. In contrast, the majority of the NPC components in yeast including the subcomplex consisting of the FG repeat-containing nucleoporins Nup49p, Nup57p and Nsp1p (Fahrenkrog et al., 1998; Rout et al., 2000), and the p62 subcomplex in vertebrates (Finlay et al., 1991; Kita et al., 1993; Guan et al., 1995) localize to both sides of the NPC and in close proximity to the midplane of the NPC. These components may constitute the core of the NPC and may compose the central transporter.

The localization of the FG repeat-containing nucleoporins throughout the NPC, in combination with *in vivo* and *in vitro* biochemical data, suggests that these proteins potentially provide multiple docking sites for the β-karyopherins throughout the NPC. While most of the FG repeat-containing nucleoporins have been shown to interact with several of the β-karyopherins, the affinity of different β-karyopherins to certain FG repeat-containing nucleoporins appears to vary. For example, overlay assays have demonstrated that the yeast Kap95p has a higher affinity for the FG repeat-containing nucleoporins Nup1p and Nsp1p than does Kap123p, which binds weakly to both nucleoporins (Rout et al., 1997). We have demonstrated a specific interaction between Nup53p and Kap121p, but not with other related and more abundant karyopherins, and will be discussed in Chapters 3 and 4. The specificity of Nup53p's interaction with Kap121p was also independently confirmed by Damelin and Silver (2000) using fluorescence resonance energy transfer (FRET) analysis. These results suggest that some nucleoporins are preferred binding sites for specific transport factors.

1.12 Non-FG Repeat-Containing Nucleoporins

Among the yeast nucleoporins, sixteen do not contain FG repeats. A number of these non-FG repeat-containing nucleoporins have also been shown to be required for nuclear transport. Mutations in any one of several genes encoding non repeat-containing nucleoporins have been shown to result in the mislocalization of poly(A)$^+$ RNA or of cNLS-containing proteins. For example, *nup120, nup84, nup133,* and *nup85* mutant yeast strains display an aberrant export phenotype as assessed by the accumulation of poly(A$^+$) RNA in the nucleus (Doye et al., 1994; Aitchison et al., 1995b; Pemberton et al., 1995; Li et al., 1995; Goldstein et al., 1996; Siniossoglou et al., 1996). Also, mutations in either *NIC96*, or *NUP82*, encoding Nic96p and Nup82p were found to affect cNLS protein import (Grandi et al., 1995a; Shulga et al., 1996; DeHoratius and Silver, 1996). However, some of these effects may arise due to pleiotropic effects of mutations in a nucleoporin. Thus, we must keep in mind the possibility that mutations of nucleoporins, which do not bind karyopherins, may indirectly affect transport.

The non-FG repeat-containing nucleoporins Nup188p, Nup192p, the integral membrane protein Pom152p, and two structurally related nucleoporins Nup157p and Nup170p, and Nic96p have been calculated to account for as much as 25% of the mass of the NPC (Aitchison et al., 1995a). The abundance of these proteins and their close proximity to the core of the NPC suggests that these are components of the repetitive structures that form the core of the NPC (Rout et al., 2000). Genetic interactions between *POM152*, *NUP188*, and *NIC96*, as well as physical interactions between the products of these genes, indicate that these abundant nucleoporins are functionally linked to one another (Aitchison et al., 1995a; Nehrbass et al., 1996; Zabel et al., 1996). In addition, the interaction of Nup170p and Nic96p with FG repeat-containing nucleoporins suggests that these may function to position the FG repeat-containing nucleoporins so that they may interact with the soluble transport machinery (Grandi et al., 1993; Kenna et al., 1996; see also Chapter 3). Taken together these results suggest that these abundant nucleoporins may play a key role in forming the foundation on which other nucleoporins are organized.

The identification of biochemical and genetic interactions between nucleoporins suggests that they are organized into subcomplexes with similar or related functions. The first subcomplex to be discovered was isolated from vertebrates and consists of the NPC core nucleoporins p62, p54, p45, and p58 (p62 subcomplex) (Dabauvalle et al., 1990; Finlay et al., 1991; Kita et al., 1993; Guan et al., 1995; Hu et al., 1996). A number of other subcomplexes have since been identified in both yeast and vertebrates (these are illustrated in *Figure 1-4*; Grandi et al., 1993; Grandi et al., 1995a; Grandi et al., 1995b; Wimmer et al., 1992; Siniossoglou et al., 1996; Macauley et al., 1995; Pante et al., 1994; Radu et al., 1995; Bastos et al., 1997; Grandi et al., 1997; Fontoura et al., 1999). Of particular interest is the finding that the yeast nucleoporins Nic96p and Nsp1p are members of two different, but related, subcomplexes indicating that some nucleoporins may have multiple functions (Nehrbass et al., 1996; Zabel et al., 1996; Grandi et al., 1993; Grandi et al., 1995a). Consistent with this idea, mutations in several nucleoporins have been shown to affect nuclear envelope structure as well as in RNA export (Wente and Blobel, 1994; Fabre et al., 1994; Doye et al., 1994; Pemberton et al., 1995; Li et al., 1995; Aitchison et al., 1995b; Heath et al., 1995; Gorsch et al., 1995; Murphy et al., 1996; Siniossoglou et al., 1996; Goldstein et al., 1996). Taken together, one must carefully consider possible pleiotropic effects when assessing the phenotype of nucleoporin mutant cells.

1.13 Alternate Roles for Nucleoporins

Yeast molecular genetics has been an invaluable tool in defining the role of individual NPC components in the structural organization of the NPC and the stability of the complex in the NE (reviewed in Doye and Hurt, 1997). Yeast strains containing a mutation in any one of several genes encoding nucleoporins have been shown to display severe structural defects of the NE. The observed phenotypes include grape-like perturbations of the NE containing NPCs (Wente and Blobel, 1993), NE herniations over NPCs (Wente and Blobel, 1993; DeHoratius and Silver, 1996; Murphy and Wente, 1996; Nehrbass et al., 1996; Zabel et al., 1996; Ho et al., 1998), gross deformations of the nucleus (Bogerd et al 1994; Aitchison et al., 1995a; Goldstein et al., 1996), formation of annulate lamellae (Wente and Blobel, 1993, Aitchison et al., 1995a), and clustered NPCs (Wente et al., 1994; Fabre et al., 1994; Doye et al., 1994; Li et al., 1995; Heath et al., 1995; Aitchison et al., 1995b; Pemberton et. al., 1995; Gorsch et al., 1995; Murphy et al., 1996; Siniossoglou et al., 1996; Goldstein et al., 1996). However, since several of the nucleoporins whose deletions cause the phenotypes described above have also been shown to functionally interact with the β-karyopherins, pleiotropic defects associated with many of these mutant strains may be indirectly caused by import and/or export defects.

Other non-NPC components have been shown to have an effect on both NE structure and function. Among these, a protein involved in fatty acid biosynthesis, acetyl-CoA carboxylase 1 (Acc1p) (Schneiter et al., 1996); an integral membrane protein of the nuclear envelope/endoplasmic reticulum, Snl1p (Ho et al., 1998); a protein required for proper spindle pole orientation, Nnf1p (Shan et al., 1997); and the endoplasmic reticular integral membrane proteins required for sporulation, Spo7p and Nem1p (Siniossoglou et al., 1998) have all been implicated in maintaining the overall structure of the NE. A mutation in *ACT2*, the gene encoding a divergent actin, has also been shown to result in defective protein import, aberrantly localized NPCs, and a general decrease in the amount of nucleoporins (Yan et al., 1997). Mutations in *NNF1* also result in an attenuation of nuclear transport (Shan et al., 1997). In addition, genetic interactions between the genes encoding the proteins mentioned above and several nucleoporins, or karyopherins, have been demonstrated (Schneiter et al., 1996; Yan et al., 1997; Ho et al., 1998; Siniossoglou et al., 1998). Clearly, the structural organization of NPCs and the NE is a highly coordinated process requiring multiple factors including the synthesis of lipids, cytoskeleton biogenesis, and the function of several nucleoporins.

1.14 Biogenesis of the Nuclear Pore Complex

A great deal has been learned about the structure and composition of the NPCs and the functional role that nucleoporins play in nucleocytoplasmic transport. However, relatively little is known about NPC assembly and the regulatory factors that control this process. Much of what we know about the assembly of NPCs comes from the analyses of higher eukaryotic cells (reviewed in Gant et al., 1998) and is restricted to assembly and disassembly events that occur during mitosis. Metazoan cells assemble intact nuclear membranes during interphase in order to accommodate growth and expansion of the NE.

However, during mitosis, the NE vesiculates, exposing the underlying chromatin to the mitotic apparatus. The NE breakdown is accompanied by the disassembly of the NPCs and the dispersion of nucleoporins into the mitotic cytoplasm. The purification of NPC subcomplexes from mitotic cells suggests that individual components of the NPC do not fully disassemble (Finlay et al., 1991; Macauley et al., 1995; Grandi et al., 1997). Although little is known about the disassembly process, the mitotic-specific phosphorylation of Nup153p, gp210, CAN/Nup214p, Nup98p, and Nup358p suggests that this modification may play a role in triggering the disassembly of NPCs (Macauley et al., 1995; Favreau et al., 1996). Late in mitosis, the nuclear envelope is reformed by fusion of NE vesicles, and NPCs are assembled at the pore membrane domain (Macauley and Forbes, 1996), presumably by the assembly of partially disassembled subcomplexes.

Field emission in-lens scanning electron microscopy (FEISEM) of reconstituted NEs has provided detailed views of NPC intermediates indicating that NPC assembly is a hierarchical, multistep process (Goldberg et al., 1997; reviewed in Gant et al., 1998). The stages of NPC assembly have been surmised to begin with the formation of 4 nm dimples in the NE. These dimples are presumably filled by a stepwise addition of individual components, or subcomplexes, which initially form the internal structures of the NPC and conclude with the formation of peripheral structures (Goldberg et al., 1997). Consistent with this ordered assembly, recent studies have begun to decipher the temporal order in which nucleoporins, or nucleoporin subcomplexes, are incorporated into the assembling NPC. Immunofluorescence microscopy of cultured mammalian cells determined that Nup153p and the integral membrane protein Pom121p were recruited early (during anaphase) to the chromatin and the sites of the reforming NEs, suggesting that these proteins may serve to seed the assembly of NPCs (Bodoor et al., 2000). This is followed by the arrival of the components of the p62 subcomplex, CAN/Nup214p and Nup84p during anaphase/telophase. Finally, the transmembrane protein gp210, and the nuclear filament component Tpr, are localized to the NEs during telophase (Bodoor et al., 2000). The arrival of the latter components may require active import, and thus functional NPCs (Bodoor et al., 2000).

1.15 Nuclear Pore Complex Biogenesis in Yeast

Whether the mitotic reassembly of NPCs faithfully reflects the events that transpire during interphase NPC assembly remains an open question. In yeast, the insertion of NPCs into the membranes must occur while the NE is intact, since the NE does not break down and NPCs do not disassemble during M-phase. As a consequence yeast provide an adequate system in which to investigate the assembly of NPCs into an intact membrane. Phenotypic analysis of yeast mutants and the molecular analysis of NPC components have identified several nucleoporins that may play a role in the assembly of NPCs. The integral membrane proteins Pom152p, Pom34p, and Ndc1p have been speculated to play a central role by anchoring the NPCs to pore membrane domain (Wozniak et al., 1994, Chial et al., 1998; Rout et al., 2000). In addition, several peripherally associated nucleoporins with possible roles in NPC biogenesis include those in which the NPC/NE is perturbed in the respective mutants (see Alternate Roles for Nucleoporins; Section 1.13). Furthermore, the characterization of novel NPC components such as the COPII coatomer protein Sec13p, and its homologue Seh1p, suggests a possible link between the NPC and NE biogenesis (Siniossoglou et al., 1996).

Mutants in which the proper recruitment of other nucleoporins to the NPC is altered suggest that those components are required for the proper assembly of NPCs. Amongst these, mutants in *NUP57* fail to incorporate Nup49p, Nsp1p, and Nup116p at the NPCs, while Nic96p, Nup82p, and others were not markedly affected, indicating that *nup57* mutants may have partially assembled NPCs (Bucci and Wente, 1998). Likewise, Nup120p was found to be essential for the recruitment of Nup85p (Goldstein et al., 1996) and Nup170p has been implicated in maintaining the proper stoichiometry of FG repeat-containing nucleoporins including Nup1p and Nup2p at the NPC (Kenna et al., 1995). A reduction in the number of NPCs was observed in temperature sensitive mutants of Nic96p (Zabel et al., 1996), and in cells depleted of Nsp1p (Mutvei et al., 1992). Further analysis of nucleoporin mutants will likely begin to resolve the nucleoporins that are essential for the biogenesis of NPCs and possibly the order in which these components are recruited to the forming NPCs.

1.16 The Focus of This Thesis

We have used a combination of genetic and biochemical approaches to identify and characterize novel nucleoporins in the yeast *Saccharomyces cerevisiae*. This thesis focuses largely on the nucleoporin Nup53p, its interactions with other components of the NPC, and its role in nucleo-cytoplasmic transport and NPC biogenesis.

Chapter 2

Materials and Methods

2.1 Yeast Strains and Media

The yeast strains used in this study are described in *Table 2-1* or referenced below. All strains were grown in YPD (1% yeast extract, 2% bactopeptone and 2% glucose) as described in Sherman et al. (1986), or in synthetic media (SM) supplemented with the necessary amino acids or nucleotides containing 2% glucose. 5-Fluo-orotic acid (5-FOA) was prepared in SM media as described in Ausubel et al. (1992). All strains were grown at 30°C unless otherwise stated. Yeast manipulations were conducted as described in Sherman et al. (1986). Competitive growth assays were performed on the yeast strain NP53/NP59-2.1 and a wild type sister haploid strain as previously described (Rout et al., 1997).

Table 2-1 Yeast Strain List

Strains	Genotype	Reference
W303	*Mata/Matα ade2-1/ade2-1 ura3-1/ura3-1 his3-11,15/his3-11,1 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100*	see Aitchison et al., 1995a
DF5	*Mata/Matα ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-1/trp1-1 leu2-3,112/leu2-3,112 lys2-801/lys2-801*	
NP53Δ	*Mata/Matα ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-1/trp1-1 leu2-3,112/leu2-3,112 lys2-801/lys2-801 nup53::HIS3/+*	see Section 2.9
NP53-B1	*Mata ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801 nup53::HIS3*	Segregant of sporulated NP53Δ
NP53-A2	*Matα ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801 nup53::HIS3*	Segregant of sporulated NP53Δ
NP59Δ	*Mata/Matα ade2-1/ade2-1 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100 nup59::HIS3/+*	see Section 2.9
NP59-21	*Matα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 nup59::HIS3*	Segregant of sporulated NP59Δ
NP59-23	*Mata ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 nup59::HIS3*	Segregant of sporulated NP59Δ
NP170-11.1	*Mata ade2-1 ura3-1 his3-11.15 trp1-1 leu2-3,112 can1-100 nup170::HIS3*	Aitchison et al., 1995a
PMY17	*Mata ade2-1 ura3-1 his3-11.15 trp1-1 leu2-3,112 can1-100 pom152::HIS3*	Wozniak et al ., 1994
NP53/NP170-11.1	*ade2 ura3 his3 trp1 leu2 nup53::HIS3 nup170::HIS3 pRS316A-NUP170 (ADE3 URA3)*	Segregant of sporulated NP53/NP170 containing pRS316A-NUP170
NP59/NP170-7c	*ade2 ura3 his3 trp1 leu2 can1 nup59::HIS3 nup170::HIS3 pRS316A-NUP170 (ADE3 URA3)*	Segregant of sporulated NP59/NP170 containing pRS316A-NUP170
NP53/NP188-6a	*ura3 his3 trp1 leu2 nup53::HIS3 nup188::HIS3 pRS316A-NUP188 (ADE3 URA3)*	Segregant of sporulated NP53/NP188 containing pRS316A-NUP188

Strains	Genotype	Reference
NP59/NP188-7a	*ade2 ade3 ura3 his3 trp1 leu2 can1 nup59::HIS3 nup188::HIS3 pRS316A-NUP59 (ADE3 URA3)*	Segregant of sporulated NP59/NP188 containing pRS316A-NUP59
NP53/NP157-77a	*ura3 his3 trp1 leu2 nup53::HIS3 nup157::URA3*	Segregant of sporulated NP53/NP157
NP59/NP157-π3	*ade3 ura3 his3 trp1 leu2 nup59::HIS3 nup157::URA3*	Segregant of sporulated NP59/NP157
NP59/PM152-AC	*ade2 ura3 his3 trp1 leu2 can1 nup59::HIS3 pom152::HIS3 pRS316A-NUP59 (ADE3 URA3)*	Segregant of sporulated NP59/PM152 containing pRS316A-NUP59
NP53/NP59-2.1	*ade2 ura3 his3 trp1 leu2 nup53:HIS3 nup59::HIS3*	Segregant of sporulated NP53/NP59
NP53PA	*Mata/Matα ade2-1/ade2-1 ura3-1/ura3-1 his3-11,15/his3-11,15 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100 nup53-pA/+ (URA3 HIS3)*	Integrative transformation of W303 with Protein A-*HIS3-URA* at the 3' end of *NUP53*
NP53pPA	*Mata ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 pRS315-NUP53-pA (LEU2)*	W303 haploid transformed with pRS315-NUP53-pA
NP59pA	*ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801 nup59-protA (spHIS5)*	Rout et al., 2000
NP59pPA	*Matα ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 nup59::HIS3 pRS315-NUP59-pA (LEU2)*	NP59-21 transformed with pRS315-NUP59-pA
NP157pA	*Mata/Matα ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-1/trp1-1 leu2-3,112/leu2-3,112 lys2-801/lys2-801 nup157-protA (URA3-HIS3)/+*	Aitchison et al., 1995a
NP170pA	*Mata/Matα ura3-52/ura3-52 his3-Δ200/his3-Δ200 trp1-1/trp1 1 leu2-3,112/leu2-3,112 lys2-801/lys2-801 nup170-protA (URA3-HIS3)/+*	Aitchison et al., 1995a
cdc15-2	*Mata ade1 ade2 ura1 his7 lys2 tyr1 gal1 cdc15-2*	see Hartwell L. et al., 1971
cdc15-2B	*Matα cdc15-2*	Segregant derived from cross of cdc15-2 and PSE1-A
cdc15-2-53	*Matα cdc15-2 pRS315-NUP53-pA (LEU2)*	cdc15-2B transformed with pRS315-NUP53-pA
cdc15-2-59	*Matα cdc15-2 pRS315-NUP59-pA (LEU2)*	cdc15-2B transformed with pRS315-NUP59-pA
cdc15/cdc15-2B	*Mata/Matα cdc15-2/cdc15-2*	Cross of cdc15-2 and cdc15-2B
cdc15-2/cdc15-2B-53	*Mata/Matα cdc15-2/cdc15-2 pRS315-NUP53-pA (LEU2)*	cdc15-2B transformed with pRS315-NUP59-pA
cdc15-2/cdc15-2B-121	*Mata/Matα cdc15-2/cdc15-2 pPS1069 (TRP1)*	cdc15-2B transformed with pPS1069 (KAP121-GFP)
123Δ–14–1:HIS3	*Matα ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801 kap123::ura3::HIS3*	Rout et al.,1997
NP53/KP123	*ura3-52 his3-Δ leu2-3,112 lys2-801 kap123:;URA3 nup53::HIS3*	Segregant derived from cross of NP53-A2 and 123Δ-14-1 (Rout et al., 1997)
NP53/KP123	*ura3-52 his3-Δ leu2-3,112 lys2-801 kap123::URA3 nup59::HIS3*	Segregant derived from cross of NP59-21 and 123Δ-14-1
KP121-41	*ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801 kap121::HIS3 pRS314-kap121-41 (TRP1)*	Leslie, D., Wozniak, R.W., and J. D. Aitchison (manuscript in preparation)
ProA5-4d	*Mata ura3-52 his3Δ200 trp1-1 leu2-3,112 lys2-801 NDC1-protA (HIS3-URA3)*	Chial, H.J. et al., 1998
GNP53	*Mata ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 pGAL-NUP53 (TRP1)*	W303 transformed with pGAL-NUP53
CNP53	*Mata ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 pCUP-NUP53 (URA3-leu2d)*	W303 transformed with pCUP-NUP53
CNP53ΔC	*Mata ura3-52 his3-Δ200 trp1-1 leu2-3,112 lys2-801 nup53::HIS3*	NP53-B1 transformed with pCUP NUP53ΔC

2.2 Transformation of Yeast Cells by Electroporation

Yeast cells were transformed with DNA by electroporation as described in Ausubel et al. (1989). Briefly, yeast strains were grown in 5 mL of YPD overnight to an O.D.$_{600}$ of 0.5-1. Cells were harvested by centrifugation, washed twice with water and resuspended in 1 mL of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM lithium acetate. The suspension was mixed gently for 45 min at 30°C. 1 M Dithiothreitol (DTT) was added to the cells to a final concentration of 20 mM and the cells were incubated as before for 15 min. Following this incubation, the cells were washed twice with 1 mL of ice cold water, and once with 1mL of ice cold 1 M sorbitol. 0.5-1 μg of plasmid DNA, or 100-200 ng of linearized DNA, was mixed with 40 μL of the electrocompetent yeast cells, and pulsed in a prechilled 0.2 cm-gap disposable electroporation cuvette at 16 kV, 25 μF, 200 Ω, in a Bio-Rad Gene Pulser. The pulsed cells were resuspended in 200 μL of ice cold 1 M sorbitol and plated onto appropriate selective media at 30°C. Transformed colonies were generally visible within 3-5 days.

2.3 Amplification of DNA by PCR

The polymerase chain reaction (PCR) was used to amplify specific DNA sequences and facilitate cloning. All PCR reactions discussed in this work were performed using the Expand Long Template PCR system (Roche, Laval, Quebec, Canada) following the manufacturer's specifications. 50-100 ng of an *S. cerevisiae* genomic DNA library, 0.5 μg of *S. cerevisiae* genomic DNA, or 100 ng of a purified plasmid containing the region to be amplified was used as template. Deoxyoligonucleotide primers were customized for each sequence and synthesized using a Beckman Oligo 1000M Synthesizer, at the Department of Cell Biology, University of Alberta.

2.4 Plasmids

The plasmids described in this paper are: pRS315, *CEN/LEU2* (Sikorski and Heiter, 1989); pRS316, *CEN/URA3* (Sikorski and Heiter, 1989); pBluescript II SK (pBS) (Stratagene, La Jolla, CA); pSB32, *CEN/LEU2*-based genomic DNA library (provided by J.D. Rine, University of California, Berkeley, CA); pGAL1, a high copy plasmid based on pRS424 [*TRP1*] containing the opposing galactose inducible promoters *GAL1* and *GAL10* (pRS244; kindly provided by S. Wente, Washington University, St. Louis, MO); pCUP1, *URA3/leu2d* (pYex-Bx; Clontech, Palo Alto, CA) a multiple copy plasmid containing the copper inducible promoter of *CUP1*; pCH1122, *CEN/URA3/ADE3* (Kranz and Holm, 1990); pCH1122-POM152 (Aitchison et al., 1995a); p4047, an isolate of the pSB32 library containing a 3.8-kbp fragment encoding the *NUP59* gene; pScBH-NUP59, a 2.2-kbp *Sca*I-*Bam*HI fragment of p4047, containing the *NUP59* open reading frame (ORF) and extending from nucleotide –164 to 2,066 (where +1 is the initiation codon) inserted into *Eco*RV-*Bam*H1 cut pRS315; pBS-NUP59, a PCR product of the *NUP59* ORF extending from nucleotide +1 to 2,930 inserted into an *Eco*RV cut pBS; pH6-NUP53, a 2.3-kbp PCR product of the *NUP53* ORF extending from nucleotide –521 to 1,756 inserted into a *Pst*I-*Bam*HI cut pRS315; pBS-NUP53, a 1.8-kbp PCR product of the *NUP53* ORF extending from nucleotide +1 to 1,756 inserted into an *Eco*RV site of pBS; pGEX-NUP53, a *Bam*HI-*Bam*HI fragment of pBS-NUP53 inserted into *Bam*HI at the 3' end of the glutathione-S-transferase (GST) ORF in pGEX-3X (Pharmacia Biotech, Uppsala, Sweden); pGEX-NUP59, a *Bam*HI-*Bam*HI fragment of pBS-NUP59 inserted into a *Bam*HI site in pGEX-4T1 (Pharmacia Biotech); pGEX-KAP121, a PCR product containing the *KAP121* ORF, amplified from genomic DNA and containing an in frame *Bam*HI site at the 5' end and a 3' *Xho*I inserted at the same restriction sites in pGEX-4T1 (Pharmacia Biotech); pRS316A, a 3.5kbp PCR product of the *ADE3* gene, using genomic DNA as a template, and inserted into the *Sac*I site in pRS316; pRS316A-NUP170 (Aitchison et al., 1995a); pRS316A-NUP188 (Nehrbass et al., 1996); pRS316A-NUP59, a 2.2-kbp *Sca*I-*Bam*HI fragment of pScBH-NUP59 inserted into the same sites of pRS316A; pNMD5-GFP, the *NMD5* ORF was subcloned into the yeast multicopy plasmid pGFP-C-FUS (*URA3*) (Niedenthal et al., 1996) in frame with the *GFP* ORF to produce an *NMD5-GFP* fusion gene (kindly provided by R. Baker, and J.D. Aitchison, University of Alberta, Edmonton, Alberta); pGAL-NUP53, a 1.8-kbp *Bam*HI-*Bam*HI fragment containing the *NUP53* ORF and 3' untranslated sequence excised from pGEX-NUP53 and inserted into a *Bam*HI site in pGAL1; pGAL-NUP59 was generated by inserting a *Bam*HI-*Bam*HI fragment derived from pBS-NUP59 into a *Bam*HI site of pGAL1; pCUP-NUP53, a *Bam*HI-*Bam*HI fragment of pGAL-NUP53 inserted into a *Bam*HI site of pCUP1; pCUP-NUP53ΔC, a 1.2-kbp PCR product containing a 75-bp deletion from the 3' end of the *NUP53* ORF inserted into the *Bam*HI site of pCUP1(Clontech); pCUP1$^{\mathrm{CEN}}$, a 240-bp *Hin*dIII-*Bam*HI fragment of pCUP1 (Clontech) containing the *CUP1* promoter inserted into a *Hin*dIII-*Bam*HI cut pRS315; pCUP-NUP53$^{\mathrm{CEN}}$, a *Bam*HI-*Bam*HI fragment in pCUP-NUP53 containing the *NUP53* ORF inserted into a *Bam*HI site in pCUP1$^{\mathrm{CEN}}$. Plasmids used for gene disruptions and protein A fusion constructs are described below in Sections 2.8, and 2.9.

2.5 Complementation and Characterization of the *POM152* Synthetic Lethal Mutant Allelic to *NUP59*

Mutants dependent on a plasmid born copy of *POM152* were isolated using the colony sectoring assay described in Aitchison et al. (1995a). This screen identified four complementation groups, three of which have been previously described (Aitchison et al., 1995a; and Nehrbass et al., 1996). The remaining group contained four mutants including one designated psl40 (*POM152* synthetic lethal number 40). To identify the wild-type allele for the psl40 mutant this strain was transformed by electroporation (see Section 2.2) with a yeast genomic DNA library inserted into pSB32. Transformants were plated onto SM-Leu and allowed to grow for 3-5 days. Three Sec$^+$/5-FOA resistant colonies were isolated and propagated. The complementing plasmid was isolated from each yeast strain as described in Ausubel et al.(1989) and amplified by shuttling into the *Escherichia coli* strain DH5α (Strathern and Higgins, 1991). Restriction analysis of the complementing plasmids showed that they contained overlapping inserts. The complementing region within these plasmids was defined by deletion analysis and tested by reintroducing them into psl40. The shortest complementing insert of 3.8 kbp, plasmid p4047, was sequenced using Sequenase (United States Biochemical Corp., Cleveland, OH) and synthetic oligonucleotides complementary to plasmid sequence flanking the insert. The insert contained a single ORF (YDL088c) encoding *NUP59*. The plasmid p4047 complemented all four mutants in this group. Each of these mutants was mated with NP59/PM152 (*nup59*Δ*pom152*Δ, see Section 2.9). The resulting diploids failed to grow on media containing 5-FOA suggesting that the mutants are allelic to *NUP59*.

2.6 Sequence Alignments

Double alignments of Nup53p (YMR153w) and Nup59p (YDL088c) and multiple alignments of the indicated regions of Nup53p (amino acid residues 164-384), *Schizosaccharomyces pombe* (120-280), *Caenorhabditis elegans* (142-229), human (87-280), mouse (87-259), *Xenopus laevis* MP44 (80-267) (Stukenberg et al., 1997), and *Arabidopsis thaliana* (106-287) were done using CLUSTALW. All the listed sequences are from the GenBank database. Sequence for *S. pombe* (these sequence data are available from GenBank/EMBL/DDBJ under accession No. Z98975), *C. elegans* (accession No. Q09601), *A. thaliana* (accession No. AC001645), *X. laevis* (MP44; accession No. U95098) were found as single sequences. Three EST sequences from human (accession No. H08487, AA160569, and W25130) and mouse (accession No. AA266826, AA413806, and W97460) were aligned and a composite sequence produced using MegAlign software from DNAStar.

2.7 Construction of *NUP59*-pA and *NUP53*-pA Chimeric genes

A *NUP59*-protein A chimeric gene was assembled by inserting an 800-bp DNA fragment encoding the IgG binding domains of *Staphylococcus aureus* protein A near the 3' end of the *NUP59* ORF. A DNA fragment encoding protein A was synthesized by PCR using the plasmid pRIT2T as the template (Pharmacia Biotech Sverige) and primers containing flanking *Sal*I sites. This DNA fragment was inserted in frame into a *Sal*I site of the *NUP59* ORF, 372 bp upstream of the stop codon in pBSNUP59. The chimeric construct was then inserted into a pRS315 plasmid. The resulting plasmid (pRS315-

NUP59-pA), despite lacking the last 124 amino acid residues of the *NUP59* ORF, was functional in all mutant backgrounds tested.

A plasmid born NUP53-protein A chimera was constructed by ligating two PCR products encoding *NUP53* (nucleotides -233 to 1,424) and protein A (an 800-bp fragment encoding five IgG binding domains flanked by in frame 5' and 3' *Bam*HI sites) into the plasmid pRS315. The resulting plasmid (pRS315-NUP53-pA) functionally complements the synthetically lethal phenotypes of the *nup170Δnup53Δ* and *nup188Δnup53Δ* double null strains (see Section 2.9).

2.8 Construction of a *NUP53*-pA Strain by Integrative Transformation

A genomic copy of the *NUP53* gene was tagged by an in frame integration of the sequence encoding the IgG binding domains of protein A at its 3' end, as previously described in Aitchison et al. (1995a). Briefly, the protein A gene and adjacent *HIS3* and *URA3* genes were amplified by PCR from the plasmid pProtA/HU (Aitchison et al., 1995a). The primers used for this reaction were designed as follows: The sense primer encodes the C-terminus of Nup53p (up to, but not including the stop codon) and continues into the first 21 nucleotides of protein A; the antisense primer contains the untranslated region of *NUP53* downstream of the stop codon, and the reverse complement of the last 24 nucleotides of the *URA3* gene. The resulting PCR product encoded for the 3' end of the *NUP53* ORF fused in frame to a 700 bp protein A sequence followed by the *HIS3* and *URA3* selectable markers and sequence complementary to the untranslated region downstream of *NUP53*. This PCR product was transformed by electroporation (Section 2.2) into diploid W303 cells and integrants selected on SM-His-

Ura media. To identify cells that synthesized Nup53-protein A, His^+Ura^+ transformants were transferred by replica plating to nitrocellulose and converted to spheroplasts by overlaying the nitrocellulose onto filter paper soaked in a solution of 1 M sorbitol, 20 mM EDTA, 50 mM DTT, containing 1 mg of Zymolyase 20T/mL for 3 h at 37°C. The spheroplasts were lysed by submerging the nitrocellulose filter in 25 mM Tris base, 192 mM glycine, and 20% (v/v) MeOH (Towbin et al., 1979) for 10 min. Filters were washed in PBS (Phosphate buffered saline; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5) containing 0.1% (v/v) Tween 20 (PBS-T) and colonies expressing protein A were detected by Western blotting (see Section 2.25) with rabbit anti-mouse IgG followed by donkey anti-mouse antibodies conjugated to horseradish peroxidase (HRP), and visualized by enhanced chemoluminescence (ECL) (Amersham Corp, Oakville, Ontario). Out of 16 His^+ Ura^+ colonies isolated, one expressing protein A was identified. Whole cell lysates (see Section 2.18) prepared from several of these colonies including the single positive were analyzed by SDS-PAGE and Western blotting to determine whether the protein A fusion migrated at the expected size of 83 kD (53 kD from Nup53p + 30kD from protein A).

2.9 Construction of *NUP59* and *NUP53* Null Mutations

Deletion of the *NUP53* and *NUP59* genes was performed by integrative transformation using the procedure of Rothstein (1991). The *NUP53* deletion constructs were prepared in pBS-NUP53 by replacing nucleotides -87 to 1,649 with the *HIS3* selectable marker gene. Similarly, *HIS3* was used to replace nucleotides -95 to 1,684 of the *NUP59* gene in the plasmid pBS-NUP59. The resulting disruption constructs were linearized by restriction enzyme digest and transformed into DF5 (for *nup53::HIS3*) or W303 (for *nup59::HIS3*) diploid yeast and integrants selected on SM-His plates. For either case, His$^+$ transformants were analyzed by Southern blotting to identify heterozygous diploids carrying the *nup53::HIS3* or the *nup59::HIS3* allele. Cells containing a null mutation were sporulated and tetrads dissected on YPD plates. In both cases all spores were viable and the *HIS3* marker segregated with the expected 2:2 ratio. Southern blotting of the His$^+$ haploid segregants confirmed the absence of the wild-type genes, and that the *nup53Δ* and *nup59Δ* haploids were viable.

2.10 Construction of Double Disruptions Strains

Double null mutants of *nup53Δ* and *nup59Δ* and each in combination with *nup157Δ*, *nup170Δ*, *nup188Δ*, and *pom152Δ* were constructed as follows: The *nup53Δ* (NP53-A2 or NP53-B1) and *nup59Δ* (NP59-23 or NP59-21) haploid strains were crossed with one another and the null haploids of *nup157Δ* (NP157-2.1; Aitchison et al., 1995a), *nup170Δ* (NP170-11.1; Aitchison et al., 1995a), *nup188Δ* (NP188-2-4; Nehrbass et al., 1996), and *pom152Δ* (PM152-75; Wozniak et al., 1994) and diploids selected by complementation of auxotrophic markers. The viability of the double null haploid strains resulting from the sporulation of the diploid strains was ensured by maintaining a complementing *URA3*-containing plasmid in all diploid strains as follows: *nup53Δnup170Δ* and *nup59Δnup170Δ* strains contained pRS316A-NUP170, *nup53Δnup188Δ* contained pRS316A-NUP188, *nup53Δpom152Δ* contained pCH1122-POM152, and the *nup53Δnup59Δ*, *nup59Δpom152Δ*, and *nup59Δnup188Δ* contained pRS316A-NUP59. Diploid strains were sporulated and tetrads dissected. The resulting haploids from all the crosses were scored for growth on SM-His media. Segregants displaying a His$^+$:His$^-$ ratio of 3:1 or 2:2 were analyzed by southern blotting to identify the spores containing the deleted copies of *nup53::HIS3*, *nup59::HIS3*, *nup157::URA3*, *nup170::HIS3*, or *pom152::HIS3* and lacking the corresponding wild type genes. The viability of the resulting double null strains was assayed by growth on 5-FOA containing media. All lethal combinations were dependent on the covering *URA3* plasmid and failed to grow on 5-FOA containing media at 30°C. The *nup53Δnup157Δ* and *nup59Δnup157Δ* were dissected without a covering plasmid. Tetrads from the *nup53Δnup157Δ* and *nup59Δnup157Δ* diploid strains lacking a covering plasmid produced four viable segregants. Double nulls were identified by scoring spores onto SM-His media and SM-Ura media. Spores that were His$^+$ Ura$^+$ were analyzed by Southern blotting to confirm the absence of the wild type *NUP157*, *NUP53*, and *NUP59* genes.

2.11 Immunofluorescence Microscopy

Immunofluorescence microscopy of yeast cells was performed as outlined in Kilmartin and Adams (1984) with modifications in Wente et al. (1992) and Aitchison et al. (1995a). Logarithmically growing cells were washed in phosphate buffer (100 mM KH_2PO_4, pH 6.8, 37.4 mM KOH) and fixed in 3.7% (v/v) formaldehyde in phosphate buffer, at room temperature. The duration of fixation was determined empirically for each epitope analyzed. Strains synthesizing protein A tagged Nup53p, and Nup59p, were especially sensitive to fixation and were treated for a maximum of 4 min. Yeast strains synthesizing Nup170pA, and Ndc1pA were fixed for 10 min. For the detection of other epitopes such as Pom152p, the FXFG repeat containing nucleoporins, Nup53p (using affinity purified anti-Nup53p antibodies), and Nup59p (using affinity purified anti-Nup59p antibodies) cells were fixed between 5-10 min. Fixed cells were washed immediately in phosphate buffer and converted to spheroplasts in an equal pellet volume of sorbitol citrate buffer (100 mM K_2HPO_4, 36.4 mM citric acid, 833 mM sorbitol) containing 0.1% (v/v) glusulase and 10 μg of Zymolyase 20T/mL for 3 h at 30°C with gentle agitation. The fixed spheroplasts were subsequently washed and resuspended in an equal pellet volume of sorbitol citrate buffer. These cells were further diluted 1:20 in sorbitol citrate buffer and spotted onto microscope slides precoated with a solution of 0.1% poly-L-lysine and allowed to settle for 5 min. Excess cells were removed and the remaining cells were permeabilized by submerging them in 100% methanol at -20°C for 4 min and immediately transferred to acetone for 30 sec at room temperature and then allowed to air dry. Fixed permeabilized cells were blocked in PBS-T containing 2% skim milk for 30 min at room temperature. Samples were probed with a primary antibody, followed by an appropriate secondary antibody in PBS-T containing 2% skim milk. Each incubation was performed for 1 h each at room temperature or overnight at 4°C in a moist environment (see Section 2.11.1). Cells were washed extensively with PBS-T containing 2% skim milk after each incubation. Following the incubation with the secondary antibody, cells were washed with PBS-T containing 2% BSA. Samples were prepared for observation by the addition of a drop of mounting medium (90% glycerol, 0.2% phenylenediamine, and 0.025% diamidino-2-phenylindole dihydrochloride (DAPI) in 0.1X PBS), covered with a coverslip, and the edges sealed with nail polish. All fluoresence images were captured using an Olympus BX-50 fluorescence microscope with a 100x oil immersion objective, SPOT digital camera and SPOT software version

2.2 (Model SP400, SPOT Diagnostics Instruments Inc.), or a Zeiss LSM501 confocal microscope. Nuclei were localized by DAPI staining.

2.11.1 Detection of Epitopes by Immunofluorescence Microscopy

Protein A fusions were detected using a preadsorbed rabbit anti-mouse IgG (1:200 dilution) (Cappel Laboratories, Organon Teknika Corp., Durham, NC) followed by preadsorbed Cy3-conjugated donkey anti-rabbit antibodies (1:400 dilution) (Jackson ImmunoResearch, West Grove, PA). The L25 NLS-β-galactosidase fusion was detected using a monoclonal antibody directed against β-galactosidase (1:200 dilution) (Roche), Pom152p using the monoclonal mAb118C3 (1:20 dilution) (kindly provided by Caterina Strambio-de-Castillia, The Rockefeller University, New York, NY), and the FXFG repeat containing nucleoporins by mAb414 binding (1:400 dilution) (BAbCo, Berkeley Antibody Co., Richmond, CA) followed by preadsorbed rhodamine-conjugated goat anti-mouse antibodies (1:400 dilution) (Jackson ImmunoResearch). The localization of endogenous and overexpressed Nup53p and Nup59p was determined using affinity purified antibodies (see Section 2.24) specific for Nup59p and Nup53p (1:2 dilution) (raised as described in Section 2.23) and detected with a Cy3-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch). Most polyclonal antibodies used for immunofluorescence were preadsorbed with an acetone powder prepared from wild type yeast cells overnight at 4°C as described in Harlow and Lane (1988) in order to reduce background staining.

2.12 Isolation of the Nup53-pA-Containing Complex

Strains expressing NUP53-pA (NP53PA) and RBP1-pA (Iouk, T.,J. Aitchison, S. Maguire, and R.W. Wozniak, manuscript in preparation) were grown in 250 mL of YPD at 30°C to a maximum O.D.$_{600}$ of 1.0. All subsequent steps were conducted at 4°C unless otherwise stated. Cells were collected by centrifugation, resuspended in 15 mL lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM MgCl, 0.2 mM PMSF, 2 μg of leupeptin/mL, 2 μg of aprotinin/mL, and 0.4 μg of pepstatin A/mL) and lysed by passaging the cells three times (at 1000ψ) in a prechilled French press chamber. The lysate was diluted with an equal volume of lysis buffer containing 40% (v/v) DMSO, and 2% (v/v) Triton X-100. The lysate was cleared by centrifugation at 11,300 x *g* for 15 min followed by filtration through a 0.2 μm-pore filter and centrifugation at 311,000 x *g* for 45 min. The cleared supernatant was incubated in batch with a bed volume of 200 μL of IgG Sepharose beads (Pharmacia) preequilibrated in wash buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1mM $MgCl_2$, 0.1% (v/v) Tween-20, 0.2 mM PMSF, 2 μg of leupeptin/mL, 2 μg of aprotinin/mL, and 0.4 μg of pepstatin A/mL) for 1 h. The bound complex was washed by repeated changes of wash buffer and eluted from the beads with 0.5 M acetic acid, pH 3.4, at room temperature, or in the case of the eluate shown in *Figure 3-3* (α-Nup59p), with 250 mM $MgCl_2$. The eluates were concentrated by lyophilization and resuspended in SDS-PAGE sample buffer. Polypeptides that copurified with Nup53pA were identified by SDS-PAGE and Western blotting using a battery of available antibodies.

2.12.1 Identification of 115 kD Component Copurifying with Nup53-pA

To identify the 115 kD copurifying polypeptide, eluates from nine immunoisolations were pooled and run on an SDS polyacrylamide gel and visualized by Coomassie blue staining. The 115 kD species was then excised from the gel. The polypeptide was digested in-gel with endopeptidase lys-C and peptides were purified and sequenced by the protein/DNA Technology Center at The Rockefeller University (New York, NY). Sequence was obtained from two peptides that correspond to amino acid residues 84-89 and 90-96 of Kap121p (YMR308c; Chow et al., 1992).

2.13 *In Vitro* Assays

2.13.1 Isolation of Recombinant Proteins

The plasmids pGEX-4T1, pGEX-NUP53, pGEX-NUP59, and pGEX-KAP121 were introduced into the *Escherichia coli* strain BLR-DE3 [F$^-$ *omp*T *hsd*S$_B$(r$_B^-$,m$_B^-$) *gal dcm*, *lon* (*srl-rec*A)306::*Tn10* (DE3)] (Novagen, Inc., Madison WI). Induction and purification of the fusion proteins were conducted according to the manufacturer's specifications (Pharmacia Biotech) with the following modifications. Expression of the fusion genes was induced at 37°C by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and further incubation for 2 h. Cells were lysed by sonication in PBS (40 O.D.$_{600}$) containing a protease inhibitor cocktail (0.1 mM PMSF, 1 µg of leupeptin/mL, 1 µg of aprotinin/mL, and 0.5 µg of pepstatin A/mL). After sonication, Triton X-100 was added to a final concentration of 1% (v/v) and the samples were clarified by centrifugation at 13,000 x *g* for 15 min at 4°C. The resulting supernatants were then allowed to bind glutathione (GT)-Sepharose beads (Pharmacia Biotech) (10 μL beads/mL of lysate). Fusion proteins were eluted with 50 mM Tris-HCl, pH 8.0 containing 10 mM reduced glutathione and a protease inhibitor cocktail. The eluted GST-Nup53p was further purified by ion exchange chromatography using a Mono-Q fast flow Sepharose column (Pharmacia Biotech) preequilibrated with 50 mM Tris-HCl, pH 8.0. The fusion protein was recovered in the flow-through. Eluates of the GT-Sepharose and the flow-through of the Mono-Q fast flow Sepharose were dialyzed against Tris-HCl, pH 8.0, and concentrated using an Ultrafree 0.5 mL centrifugal filter (Millipore, Bedford, MA). For binding studies using recombinant Kap121p, Kap121p was released from GST-Kap121p by cleavage with thrombin. GST-Kap121p (10 μg) bound to GT-Sepharose, prepared as described above, was treated with 1.5 NIH U of human thrombin (Sigma, St. Louis, MO) in PBS for 3 h at room temperature. The released Kap121p was used directly for binding to GST-Nup53p (see Section 2.13.2), which is insensitive to thrombin.

2.13.2 Soluble Binding Assays

In vitro binding assays were performed using the recombinant proteins GST-Nup53p, GST-Nup59p, Kap121p, and GST purified as described above. NEs derived from 10 O.D.$_{600}$ units of nuclei expressing protein A-tagged Nup53p, and Nup170p were extracted with 150 μL of 20 mM Tris-HCl, pH 7.5, 1 M NaCl, 0.1 mM $MgCl_2$, 1 mM DTT, 1% (v/v) Triton X-100, 0.2 mM PMSF, 2 μg of leupeptin/mL, 2 μg of aprotinin/mL, 0.4 μg of pepstatin A/mL for 15 min on ice. The extracted NEs were collected by centrifugation at 100,000 x *g* in a Beckman TLA120 rotor for 30 min at 4°C.

150 µL of the supernatant was diluted with 850 µL of extraction buffer lacking NaCl and Triton X-100. 200 µL of this diluted extract was incubated with 10 µL of GT-Sepharose beads previously loaded with 1-3 µg of the indicated GST-fusion protein in siliconized 0.5 mL tubes and incubated for 1 h on ice. Alternatively, preloaded beads were incubated with 400 µL of yeast cytosol prepared as previously described by Rout et al. (1997) (also see Section 2.16) from a yeast strain expressing Kap121-pA (PSE1-A), and diluted two-fold in PBS-T containing 0.2% casaminoacids (Difco Laboratories, Detroit, MI). After binding, the beads were washed three times with buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM $MgCl_2$, 1 mM DTT, 1% (v/v) Triton X-100, 0.2 mM PMSF, 0.4 µg of pepstatin A/mL) for Nup53-pA and Nup170-pA samples, or buffer B (20 mM Hepes, pH 6.8, 150 mM KOAc, 2 mM $Mg(OAc)_2$, 2 mM DTT, 0.1% Tween-20, 0.2 mM PMSF, and 0.4 µg of pepstatin A/mL) for Kap121-pA samples. Bound proteins were eluted with SDS sample buffer. Load fractions were prepared for SDS-PAGE by the addition of an equal volume of 2x SDS-sample buffer. For these experiments the load fractions shown in *Figure 3-4* were derived from 10% of the total extract used and the eluates represent 30% (*Figure 3-4A*) and 10% (*Figure 3-4B*) of the total eluate analyzed. Similar approaches were also used to examine the binding of recombinant Kap121 (purified as described above) to GST-Nup53p and GST alone. In these experiments the binding step was performed with 1 µg of Kap121p in 0.5 mL of buffer B.

2.13.3 Release of Kap121p from Nup53p by RanGTP

Ran-induced release of Kap121p from a preformed Kap121p-Nup53p complex and the Nup53p-containing complex purified from yeast was examined as follows. Yeast

Ran (Gsp1p; provided by Monique Floer, The Rockefeller University, New York, NY) was loaded with GTP, GDP, or GTP-γ-S exactly as described in Rexach and Blobel, (1995). Briefly, 15 μg of Ran in 10 μL of buffer B was incubated with 15 mM EDTA, 0.6 mM of GTP, GDP, or GTP-γ-S, and 2 mM DTT, for 90 min at room temperature. Following this incubation, magnesium acetate was added to the Ran suspension to a final concentration of 30 mM and the reaction incubated on ice for 15 min. The Kap121p/GST-Nup53p complex was assembled on GT-Sepharose beads as described in Section 2.13.1. The Nup53p-containing complex, bound to IgG beads, was isolated from NP53PA yeast cells as described above (Section 2.12) and washed with 0.5 mM ATP in buffer B for 30 min at room temperature before treatment with Ran. The beads (20 μL) were then incubated with 1 mM GTP alone or 0.8 μg of Ran-GTP, Ran-GDP, or Ran-GTPγS in buffer B for 30 min at room temperature. The supernatant, containing released proteins, was collected and prepared for SDS-PAGE by the addition of an equal volume of 2x sample buffer. The beads were washed several times with buffer B to remove residual unbound polypeptides. Bound proteins were eluted from the GT-Sepharose beads by the addition of SDS-sample buffer, or from the IgG beads with 0.5 M acetic acid, pH 3.4, concentrated by lyophilization, and resuspended in SDS-sample buffer. Polypeptides in bound and released fractions were resolved by SDS-PAGE and analyzed by Western blotting or silver staining.

2.13.4 Overlay Assays

Polypeptides in an enriched fraction of yeast NPCs were fractionated by SDS-hydroxylapatite chromatography as previously described (Wozniak et al., 1994).

Polypeptides in fractions 27 and 29 were separated by SDS-PAGE and transferred to nitrocellulose (for a complete profile of these fraction see *Figure 3* in Wozniak et al., 1994). Alternatively, purified recombinant GST-Nup53p and GST-Nup59p were used. Nitrocellulose membranes were blocked with transport buffer (20 mM Hepes, pH 7.5, 110 mM KOAc, 2 mM $MgCl_2$, 0.1% (v/v) Tween 20, 0.2 mM PMSF, 2 μg of leupeptin/mL, 2 μg of aprotinin/mL, and 0.4 μg of pepstatin A/mL) and probed with a cytosolic fraction isolated from yeast expressing protein A-tagged Kap121p, Kap123p, or Kap95p as previously described (see Section 2.16; Aitchison et al., 1996; Rout et al. 1997). Kap95-pA cytosol was kindly provided by Michael Rout (The Rockefeller University, New York, NY). Binding of the protein A tagged fusions was detected with HRP-conjugated donkey anti-rabbit antibodies and ECL (Amersham Corp).

2.14 Cellular Localization of Nmd5-GFP, Kap121-GFP, and GFP-Nup49p

2.14.1 Distribution of Kap121-GFP in *nup53Δ* and *nup59Δ* Null Strains

Yeast strains containing pPS1069 (KAP121-GFP [*TRP1*]; Seedorf and Silver, 1997; kindly provided by Pamela Silver, Dana Farber Cancer Institute, Boston, MA) or pUN100-GFP-NUP49 (GFP-NUP49 [*LEU2*]; Belgareh and Doye, 1997; provided by Valerie Doye, Institut Curie, Paris, France) were grown in the SM-Trp or SM-Leu media at the indicated temperatures. The cellular distribution of Kap121-GFP, and GFP-Nup49p was examined directly by fluorescence microscopy. Strains containing pNMD5-GFP were grown overnight to mid-log phase in SM-Ura -Met to induce the expression of the *NMD5-GFP* fusion gene. After washing the cells twice with water, the cellular distribution of Nmd5-GFP was directly visualized by fluorescence microscopy.

2.14.2 Cell Cycle Dependent Localization of Kap121p and Nmd5p

To examine the distribution of Kap121-GFP and Nmd5-GFP at different stages of the cell cycle in wild type DF5 cells the following procedure was used. A DF5 strain containing pPS1069, or pNMD5-GFP was grown to mid log phase in synthetic media as described above. Cells were collected by centrifugation, resuspended in water, and briefly sonicated. Nonbudded, small budded (< 70% of the diameter of the mother cell), and large budded (>70% of the diameter of the mother cell) cells were randomly identified by phase microscopy and then visually scored for the presence or the absence of a NE-associated Kap121-GFP, or Nmd5-GFP signal by fluorescence microscopy in the fluorescein channel.

2.15 Expression of *NUP53*-pA and *NUP59*-pA in *cdc15-2* Strains

The *S. cerevisiae* cell cycle mutant cdc15-2 (Hartwell, 1971) was separately transformed with the plasmid pRS315-NUP53-pA and pRS315-NUP59-pA. The resulting strains, *cdc-15-2-53* and *cdc15-2-59*, were arrested in M phase as follows. Cells were grown in SM-Leu to an O.D.$_{600}$ of approximately 0.5 at the permissive temperature of 23°C, transferred to YPD media for an additional 2-3 h, and then shifted to the non-permissive temperature of 37°C for 3.5 h. At this point, greater than 95% of the cells arrested in M phase as determined by bud size and position of the nuclei. The cell cycle block was released by shifting the cultures back to 23°C. 1 mL aliquots were harvested from asynchronous and arrested cultures at various time points after release. Cells were collected by centrifugation, resuspended in SDS sample buffer, and sonicated. For calf intestinal alkaline phosphatase (CIAP) treatments, these samples were diluted with water to 0.5% SDS and digested with 0.15 U of enzyme/µL at 37°C for 45 min. Polypeptides in the extracts then were separated by SDS-PAGE, transferred to nitrocellulose, and the protein A fusions visualized by Western blotting and ECL (Amersham Corp.).

2.16 Yeast Subcellular Fractionation

2.16.1 Isolation of Nuclei

Nuclei were isolated as described in Strambio-de Castillia et al. (1995) with the following modifications. In general, 4×10^{10} cells were grown overnight in a 2 L culture of YPD. Cells were harvested by centrifugation, washed, and resuspended in 2.2 mL of 1.1 M sorbitol/gm of cells, and converted to spheroplasts by the addition of 166µL of glusulase/g of cells and 44µL of 10 mg of Zymolyase 20T/mL/g of cells for 3 h at 30°C. Spheroplasts were washed thoroughly and lysed by homogenizing three times with a Polytron (Polytron Inc. Tallahassee, FL) in 40 mL of polyvinylpyrrolidone (PVP) solution (8% PVP, 20 mM potassium phosphate buffer, pH 6.5, 0.75 mM $MgCl_2$) containing 0.3 M sucrose and 0.001 volumes of P-solution (18 mg of PMSF /mL and 0.3 mg of pepstatin A/mL). The lysate was separated into a cytosolic fraction and a crude nuclear pellet fraction by centrifugation at 10,000 x *g* for 15 min at 4°C. This cytosolic fraction was used for soluble binding assays (Section 2.13.2) and overlay assays (Section 2.13.4). The crude nuclei were resuspended in 10.5 mL of 2.125 M sucrose in PVP solution and overlayed onto a three step sucrose gradient (consisting of 8 mL each of 2.15

M sucrose, 2.25 M sucrose, and 2.52 M sucrose all in PVP solution). The loaded gradients were centrifuged at 103,000 x *g* for 4 h at 4°C in a Beckman SW28 rotor. After centrifugation, a band at the 2.15-2.25 M sucrose interface containing the enriched nuclei was collected and stored at –70°C.

2.16.2 Isolation of Yeast Nuclear Envelopes

Nuclear envelopes were prepared as described in Strambio-de-Castillia et al. (1995) from enriched nuclei with the following modifications. All solutions contained a 0.001 volume of P-solution. Nuclear fractions were initially diluted 5-10 fold in PVP solution and pelleted by centrifugation at 13,000 x *g* at 4°C for 45 min. The resulting supernatant was decanted and the pellet resuspended in 0.25-0.5 mL of bt-buffer (bisTris-HCl, pH 6.5, 0.1 mM $MgCl_2$) and dounced to produce a homogenous suspension. DNase was added to the isolated nuclei to a final concentration of 20 µg/mL and the nuclei were incubated for 15 min at room temperature. 10 mL of bt-buffer containing 2.1 M sucrose, and 20% Nycodenz was added to the lysed nuclei, thoroughly mixed, and overlayed with a three step sucrose gradient (12 mL each of 2.0 M sucrose, and 1.5 M sucrose in bt-buffer and 2 mL of bt-buffer alone). The gradient was centrifuged at 103,000 x *g* for 24 h at 4°C. Nuclear envelopes were collected from the 1.5 M-2.0 M sucrose interface and stored at –70°C.

2.16.3 Pre-Embedding Labeling of Nuclear Envelopes

NEs for immunoelectron microscopy studies were prepared as described by Strambio-de-Castillia et al.(1995) from strains synthesizing Nup53-pA, Nup59-pA, Nup157-pA, and Nup170-pA. Pre-embedding labeling was performed as described by

Kraemer et al. (1995), and Nehrbass et al. (1996). Briefly, NEs isolated from strains synthesizing protein A tagged Nup53p, Nup59p, Nup157p, and Nup170p were diluted three fold in bt-DMSO (10 mM bisTris-HCl, pH 6.5, 0.1mM $MgCl_2$, and 20% (v/v) Me_2SO) and sedimented to the bottom of a 96-well microtitre well by centrifugation at 11,500 x *g* for 30 min at 4°C. Each well was pre-treated with 2.5% gluteraldehyde for 10 min, washed twice with water, and followed by a 15 min incubation with a 0.1% poly-L-lysine solution at room temperature. Sedimented NEs were fixed with 3.7% (v/v) formaldehyde in bt-DMSO for 5 min at room temperature and blocked in IEM buffer (5 mg of BSA/mL, 0.5X PBS, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1mM $ZnCl_2$, 0.02% NaN_3, 0.2 mM PMSF, 2 μg of leupeptin/mL, 2 μg of aprotinin/mL, and 0.4 μg of pepstatin A/mL) for a further 5 min at room temperature. Rabbit anti-mouse IgG (Cappel) was diluted 1:100 in IEM buffer, added to the wells and incubated overnight at 4°C. Following this incubation NEs were washed by three changes of IEM buffer. Binding was detected by a 1:10 dilution of anti-rabbit IgG conjugated to 5 nm (Nup53-pA) or 10 nm (Nup59-pA, Nup157-pA, and Nup170-pA) gold particles (Sigma) in IEM buffer for 1 h at room temperature. Wells were washed again to remove unbound secondary antibodies in 100 mM potassium phosphate, pH 6.5, 1 mM $MgCl_2$, and fixed for 12 h with 2.5% (v/v) gluteraldehyde in 100 mM cacodylate buffer, pH 7.2, at 4°C. The NEs were dehydrated and processed for electron microscopy (Wray and Sealock, 1984; Rout and Kilmartin, 1990). Decorated NPCs that were clearly sectioned perpendicular to the plane of the NE were photographed and the distance from the center of the gold particle to the midplane of the associated NPC was quantitated with a microtome (Kraemer et al., 1995).

2.17 Induction of *NUP53* Overexpression

For the induction of *NUP53* and *NUP59* under the control of the *GAL1* promoter, cells containing the appropriate pGAL1-based plasmid (pGAL-NUP53, or pGAL-NUP59) were grown to mid-logarithmic phase in SM-Trp containing 2% glucose, washed extensively with water, and transferred to SM-Trp containing 2% galactose as the sole carbon source, at 30°C for the indicated times. Induction of *NUP53* in strains carrying pCUP-NUP53, and pCUP-NUP53^{CEN} was achieved by the addition of copper sulfate to a final concentration of 0.5 mM into the growth media at 30°C for the indicated times.

2.18 Time Course of *NUP53* Overexpression

For the time course of *NUP53* expression GNP-NUP53 and CNP-NUP53 cells were collected by centrifugation prior to, or at the indicated time after the start of induction. Whole cell lysates from both induced and uninduced cultures were prepared as follows: a 50 μL pellet of yeast cells was harvested by centrifugation and resuspended in 875 μL of 0.25 N NaOH, 1% (v/v) β-mercaptoethanol and incubated for 10 min on ice. Polypeptides in these samples were precipitated by the addition of 50% trichloroacetic acid (TCA) to a final concentration of 12% (125 μL) and incubated for 10 min at 4°C. Polypeptides were collected by centrifugation at 15,000 x *g* for 10 min at 4°C. The resulting supernatant was decanted and the pellet washed in 90% (v/v) methanol, and air dried. The dry pellet was resuspended in 150 μL of SDS sample buffer, sonicated briefly and heated at 60°C for 30 min. Polypeptides in these samples were separated by SDS-PAGE, and analyzed by immunoblotting and ECL (Amersham Corp.).

2.19 Localization of Pho4p-GFP in *kap121* Mutant Cells and Cells Overexpressing *NUP53*

Wild type DF5 yeast cotransformed with pCUP1, or pCUP-NUP53, as well as a plasmid encoding the NLS (amino acid residues 140-166) of Pho4p fused to *GFP* (Pho4-GFP [*TRP1*]; Kaffman et al., 1998a; kindly provided by Erin O'Shea, University of California, San Francisco, CA), or a plasmid encoding the NLS of the large T antigen of the SV40 in frame with the *GFP* ORF (cNLS-GFP [*LEU2*]; Shulga et al., 1989; kindly provided by David Goldfarb, Rochester University, Rochester NY) were grown in SM-Ura-Trp or SM-Ura-Leu to mid-logarithmic phase at 30°C. The distribution of Pho4-GFP and cNLS-GFP was visualized directly by fluorescence microscopy in the fluorescein channel prior to, and after a 6 h induction in the presence of 0.5mM $CuSO_4$.

kap121-41 mutant cells expressing PHO4-GFP or cNLS-GFP were grown to mid-logarithmic phase at 23°C in SM-Trp or SM-Leu media. The distribution of Pho4-GFP and cNLS-GFP was assessed by direct fluorescence microscopy as indicated above.

2.20 Ultrastructural Analysis of Yeast Cells

2.20.1 Preparation of Yeast Cells Using Potassium Permanganate

Wild type W303 cells containing pGAL-NUP53, or pGAL1, were harvested by centrifugation at the indicated times after induction. Samples were fixed and stained in 1.5% $KMnO_4$ for 20 min at room temperature (Nuttley et al., 1994). Fixed cells were subsequently incubated in 1% sodium periodate for 20 min, followed by 1% NH_4Cl for

10 min at room temperature, and contrasted with 2% aqueous uranyl acetate overnight at 4°C. Samples were dehydrated by consecutive 10 min incubations through a graded ethanol series (consisting of 60%, 80%, 95%, and 100% ethanol), followed by three washes in propylene oxide. Fixed and dehydrated cells were embedded in TAAB 812 resin. Ultra-thin sections were examined in a Phillips 410 transmission electron microscope.

2.20.2 Preparation of Yeast Cells by Gluteraldehyde Fixation

In order to observe protein structures by electron microscopy wild type cells containing the pGAL-NUP53 plasmid were induced for 16 h as indicated above and processed for electron microscopy as described in Byers and Goetsch, (1991) and Wente et al. (1992) with the following modifications. Cells were fixed in phosphate citrate buffer (170 mM KH_2PO_4, 30 mM sodium citrate, pH 5.8) containing 1.85% (v/v) formaldehyde, and 2% (v/v) gluteraldehyde for 2 h at room temperature. Cells were washed and resuspended in 100 mM Tris, pH 9.4, 10 mM DTT for 10 min at 30°C and converted into spheroplasts in phosphate citrate buffer containing 10 % (v/v) glusulase and 10 μg of Zymolyase 20T/mL for 3 h at 30°C. After several washes in phosphate citrate buffer the spheroplasts were swelled for 15-30 min at room temperature in 0.1 M NaAc, pH 6.1, and refixed for 15 min. The cells were washed three times in 0.2 M cacodylate buffer, pH 7.2 and then post-fixed in 1% osmium tetroxide in 0.2 M cacodylate buffer, pH 7.4, on ice for 1 h. Cells were contrasted in 2% aqueous uranyl acetate overnight at 4°C, and dehydrated by passaging through ethanol. Cells were embedded in TAAB 812 resin and ultrathin sections examined by electron microscopy.

2.21 Post-Embedding Immunoelectron Microscopy

Immunoelectron microscopy was performed as outlined in Wente et al. (1992). Wild type W303 cells carrying pGAL-NUP53, or pGAL1, were induced 9.5 h at 30°C in SM-Trp containing 2% galactose, with an additional 2 h incubation in YPG (1% yeast extract, 2% bactopeptone, 2% galactose) at the same temperature. Cells were harvested by centrifugation and spheroplasted in 1.1M sorbitol containing 10% (v/v) glusulase and 10 μg of Zymolyase 20T/mL for 3 h at 30°C. Spheroplasts were swelled in 0.6 M sorbitol for 15 min. Cells were collected by centrifugation and the pellet fixed in 0.1 M cacodylate buffer, pH 7.25, containing 0.075% (v/v) gluteraldehyde, and 4% formaldehyde for 1 h at 4°C with continuous mixing. Fixed spheroplasts were collected by centrifugation at 1000 x *g* for 5 min and dehydrated by consecutive 10 min washes in 50%, 70%, and 90% (v/v) ethanol. Dehydrated cells were collected by centrifugation and the pellet resuspended in LR-White resin containing 20% ethanol and incubated for 1 h at RT. Cells were collected by centrifugation and the pellet resuspended in 100% LR-White resin. This resin was allowed to infiltrate the cells overnight at 4°C protected from light with continuous end-over-end mixing. Polymerization of the resin was induced by exposure to UV irradiation for 24 h at 4°C. Thin sections derived from these samples were mounted onto nickel grids coated with formvar, and coated with carbon. To detect Nup53p, the cells were blocked for 5 min with PBS-T containing 20 mM glycine and 10 min in PBS-T containing 2% BSA (PBS-T/BSA) at room temperature. After repeated washes with PBS-T/BSA the grids were probed with affinity-purified antibodies to Nup53p at a 1:2 dilution in PBS-T/BSA overnight at 4°C followed by a series of washes in PBS-T/BSA. The sections were then incubated with goat anti-rabbit antibodies conjugated to 10 nm gold (Sigma, St. Louis, Mo.) at a 1:20 dilution for 1 h at RT. Samples were washed in PBS-T/BSA and refixed for 5 min in PBS containing 2% (v/v) gluteraldehyde, washed with water, and contrasted by staining with 2% aqueous uranyl acetate for 10 min at room temperature. Samples were visualized by electron microscopy.

2.22 Sodium Carbonate Extractions of Yeast Nuclei and Nuclear Envelopes

Yeast nuclear envelopes isolated form CUP53 and CUP1 (induced for 8 h). Crude nuclei fraction was isolated from *nup53Δ* cells containing either pCUP-NUP53 or pCUP-NUP53ΔC (induced for 8 h) as previously described (Section 2.16; Tcheperegine et al., 1999). Sodium carbonate extractions were performed as previously described (Wozniak et al. 1994). Briefly, approximately 0.25 mg NEs in 10 mM bis-Tris, pH 6.5, 0.1 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 2 μg of leupeptin/mL, 2 μg of aprotinin/mL, and 0.4 μg of pepstatin A/mL were dounced several times to homogeneity. An equal volume of 0.2 M sodium carbonate, pH 11.4, or buffer alone, and incubated for 15 min at room temperature. Soluble proteins were separated from NE membranes by centrifugation at 109,000 x *g* in a Beckman TLA120.2 rotor for 30 min at 4°C. The supernatant was collected and the proteins in this fraction precipitated in 10% TCA. The precipitates were washed in 90% (v/v) methanol and air dried. The precipitate and the membrane pellet were solubilized in SDS-sample buffer. The proteins in these samples were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies.

2.23 Production of Antibodies

Purified GST-Nup53p, GST-Nup59p, and GST-Kap121p were used to elicit an immune response in rabbits. Approximately 300 μg of the appropriate purified GST-protein fusion (see Section 2.13.1) was emulsified in Freund's complete adjuvant and injected subcutaneously into rabbits. Booster injections were administered every 4 to 6 weeks as described above except that Freund's incomplete adjuvant was used. Samples of blood were collected ten days after each booster injection and the presence of specific antibodies was assayed by probing a complex mixture of polypeptides (containing the target protein) separated by SDS-PAGE by Western blotting and ECL (Amersham Corp.).

2.24 Affinity Purification of Antibodies

Polyclonal antibodies directed against Nup53p, Nup59p, and Nup170p (Nehrbass et al., 1996) were affinity purified from sera using a previously described procedure (Harlow and Lane, 1988) modified as follows. Purified fractions of GST-Nup170p, GST-Nup59p, and GST-Nup53p were separated by SDS-PAGE and transferred to nitrocellulose. The full length fusions were detected by amido black staining, excised and blocked in PBS-T containing 5% skim milk for 1 h at room temperature. The nitrocellulose strips were incubated with the appropriate serum at a 1:500 dilution in PBS-T containing 5% skim milk for 1 h at room temperature. Probed strips were washed extensively in PBS-T to remove unbound antibodies. Bound antibodies were eluted with 0.1 M glycine, pH 2.5, and immediately neutralized by the addition of 1M Tris-HCl, pH 8.0 to a final concentration of 100 mM. Affinity purified antibodies were used diluted at

1:10 ratio for Western blots or diluted at a 1:2 ratio for immunofluorescence microscopy (see Section 2.11.1) and immunoelectron microscopy (see Section 2.21) in PBS-T containing 2% skim milk.

2.25 Immunoblotting

The detection of various proteins by Western blotting was conducted as follows. Protein samples separated by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes. Transferred protein profiles were visualized by amido black staining and then blocked with PBS-T containing 5% skim milk for 1 h at room temperature. Blocked membranes were probed for 1 h at room temperature or overnight at 4°C with the appropriate primary antibody in PBS-T containing 5% skim milk, or PBS-T containing 2% BSA, at the indicated dilution. Protein A fusions were detected with rabbit anti-mouse IgG (at a 1:2500 dilution) (Capell; Organon Technika Corp. West Chester, PA). The GLFG repeat containing nucleoporins Nup116p, Nup100p, Nup57p, and the N-terminus of Nup145p were detected with mAb192 (1:20) (Wente et al., 1992). Nup170p in *Figure 3-3*, was detected with affinity-purified anti-Nup170 polyclonal antibodies (1:5) (Nehrbass et al., 1996). Polyclonal sera, produced as described above (Section 2.23), was used to detect Nup53p (1:2500), Nup59p (1:2500), and Kap121p (1:2500). GST and GST fusions were detected with polyclonal anti-GST antibodies (1:2000 dilution) (Molecular Probes, Eugene, OR), and the endoplasmic reticulum protein, Kar2p, with polyclonal antibodies directed against *S.cerevisiae* Kar2p (1:10,000; kindly provided by R. Rachubinski, University of Alberta, CA). Antibody binding was visualized with HRP conjugated donkey anti-rabbit (1:5000), or HRP conjugated goat anti-mouse (1:5000) and ECL using procedures outlined by the manufacturers (Amersham Corp.).

Chapter 3

Specific Binding of the Karyopherin Kap121p to a Subunit of the Nuclear Pore Complex Containing Nup53p, Nup59p, and Nup170p

3.1 Overview

Two previously uncharacterized yeast nucleoporins were found through a genetic screen aimed at identifying proteins that genetically interact with *POM152*. These structurally related nucleoporins have been termed Nup53p and Nup59p. An NPC subcomplex containing Nup53p, Nup59p, and Nup170p was affinity purified from cells expressing a functional Nup53-pA. All of these components were found to be distributed symmetrically on both faces of the NPC. In addition, the karyopherin Kap121p was also associated with the Nup53p-containing subcomplex. Using *in vitro* binding assays we found that all of the nucleoporins interact with each other, but the interaction with Kap121p is directly mediated by Nup53p. This interaction was found to be specific for Kap121p as other more abundant karyopherins did not bind Nup53p in several different assays. This interaction can be disrupted by the GTP bound form of the small GTPase Ran. The physiological relevance of this interaction is further underscored by the observations that *NUP53* mutations altered the subcellular distribution of Kap121p, and the Kap121p-mediated import of a ribosomal L25-reporter protein. Interestingly, Nup53p is specifically phosphorylated during mitosis. This modification correlates with a transient, reduction in the perinuclear localization of Kap121p.

3.2 Identification of Nup53p and Nup59p

We have previously used a synthetic lethal screen to identify proteins that interact with the nuclear pore-membrane protein Pom152p (Aitchison et al., 1995; Nehrbass et al., 1996). Three of the four complementation groups identified by this screen have been characterized and shown to contain mutants allelic to three nucleoporin genes, *NUP170*, *NUP188*, and *NIC96*. Like Pom152p, the products of each of these genes are abundant constituents of the NPC. A fourth complementation group has been identified which contains four mutants that are allelic to a gene encoding a 528 amino-acid residue polypeptide (termed YDL088c). This gene has also been previously identified as a multicopy anti-suppressor of a temperature-sensitive mutant of DNA polymerase δ and was termed *ASM4* (Giot et al., 1995). However, the function of *ASM4* was undefined.

A comparison of the YDL088c ORF with the remainder of the yeast genome identified a second ORF, YMR153w, of 475 amino-acid residues which exhibits a high degree of sequence similarity (30% identity, 48% similarity; *Figure 3-1A*). Further comparisons of these two sequences to available data bases identified structurally similar ORFs in *Schizosaccharomyces pombe*, human, mouse, *Xenopus laevis*, *Caenorhabditis elegans* and *Arabidopsis thaliana* suggesting these sequences are highly conserved in all eukaryotes. In each case, the metazoan sequences are most similar to the YMR153w ORF. This is highlighted by a block of amino-acid residues within the central third of the polypeptides (*Figure 3-1B*).

To determine the subcellular localization of the YDL088c and YMR153w gene products, both genes were tagged by the insertion of a DNA fragment encoding the IgG-binding domains of *S. aureus* protein A at (YMR153w) or near (YDL088c) the 3' end of their ORFs. These chimeric genes were each expressed in yeast and the resulting fusion proteins were localized by indirect immunofluorescence (*Figure3- 2A*). In both cases, the fluorescent signal was visible along the nuclear surface in a punctate pattern characteristic of that observed for nucleoporins. Their co-purification with isolated NEs, their presence in an enriched fraction of yeast NPCs (see *Figure 3-5*), and

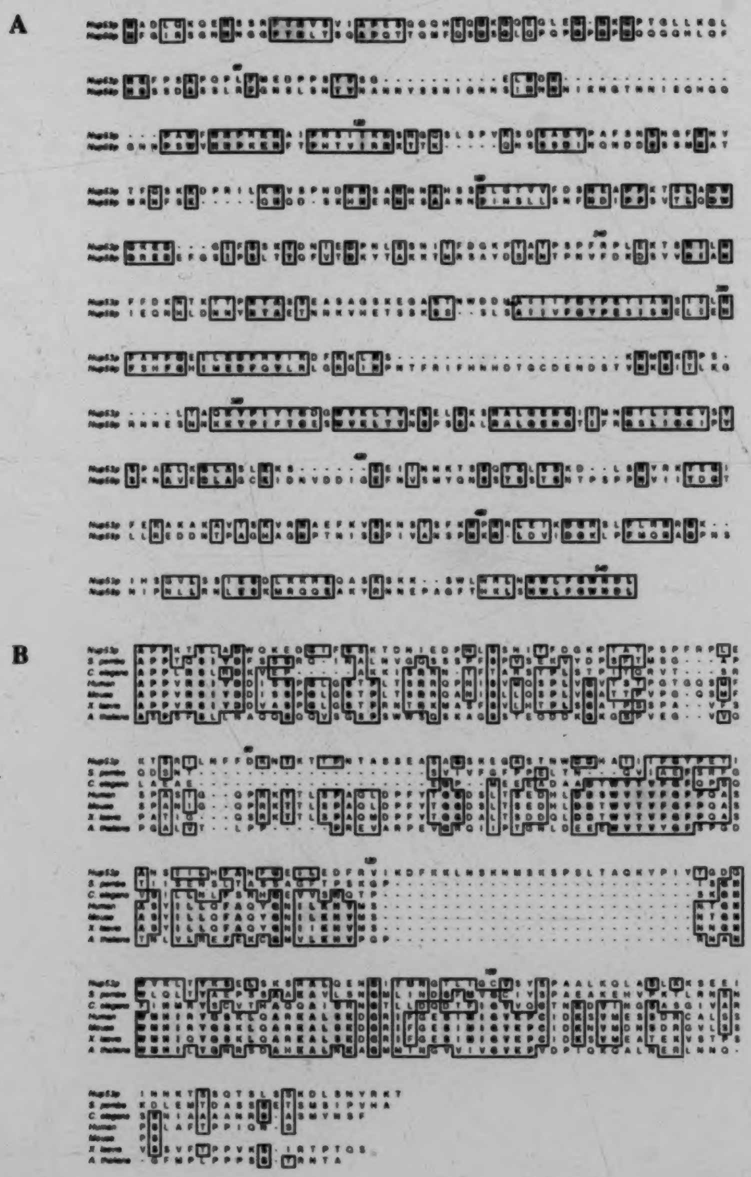
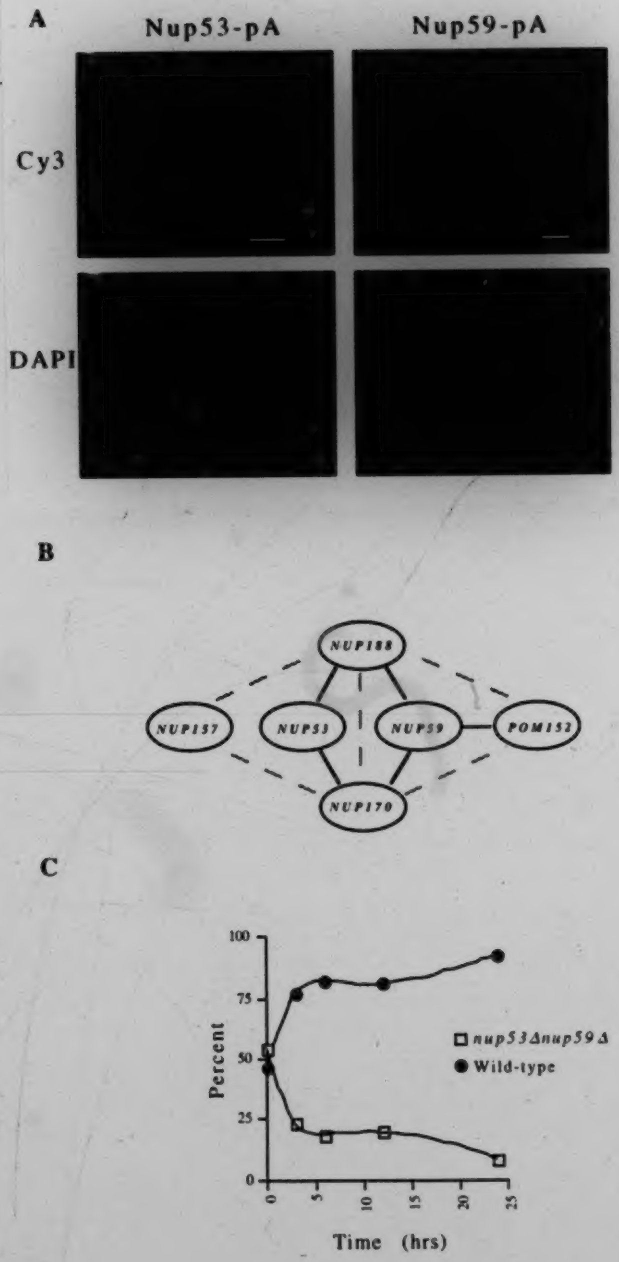


Figure 3-1. Sequence alignment of the yeast ORFs YMR153w, YDL008c and multiple metazoan homologues. Shown in panel A is the sequence alignment of YMR153w (Nup53p) and YDL008c (Nup59p) produced using CLUSTALW. A database search with Nup53p identified potential homologues in yeast and metazoan species including *Schizosaccharomyces pombe* (S. pombe), human, mouse, *Xenopus laevis* (MP44; X.laevis), *Caenorhabditis elegans* (C.elegans) and *Arabidopsis thaliana* (A.thaliana). The central third of these ORFs shows the highest degree of cross species similarity. An alignment of these regions was performed with CLUSTALW and is shown in panel B. Similar and identical residues are shaded and boxed. Residues that are identical in four or more sequences are shown in bold. Position numbers are indicated above the sequence alignment.

immunoelectron microscopy of isolated NEs (see *Figure3- 9*) further supported that these proteins are nucleoporins,. On the basis of these data, we have named the products of the YMR153w and YDL088c ORFs Nup53p and Nup59p, respectively, in accordance with established nucleoporin nomenclature and representing their deduced molecular masses.

To examine the phenotypes of *nup53Δ* and *nup59Δ* null mutations we replaced the *NUP53* and *NUP59* ORFs with the *HIS3* gene by integrative transformation of the diploid strains W303 and DF5. Sporulation and tetrad analysis of the resulting heterozygous diploid strains (NP53Δ and NP59Δ) yielded four viable haploids exhibiting a 2:2 segregation of the *HIS3* marker. These results indicate that the null mutations *nup53Δ* and *nup59Δ* are viable. Both the *nup53Δ* (NP53-B1) and the *nup59Δ* (NP59-21) haploid strains grew at or near wild type rates at temperatures ranging from 16°C to 37°C. This phenotype is similar to that observed in strains lacking *NUP157*, *POM152*, and the *POM152*-interacting genes *NUP170* and *NUP188* (Wozniak et al., 1994; Aitchison et al., 1995; Nehrbass et al. 1996; Zabel et al., 1996; Kenna et al., 1996). However, double null combinations of *nup59Δ* with either *nup170Δ*, *nup188Δ*, or *pom152Δ* were synthetically lethal. Similarly, *nup53Δ* in combination with *nup170Δ* and *nup188Δ* also exhibited a lethal phenotype. However, *nup53Δnup157Δ*, *nup59Δnup157Δ*, and *nup53Δnup59Δ* haploids were viable, although the latter two exhibited growth defects at temperatures ranging from 23°C-37°C. These results are summarized in *Figure3- 2B*. The growth defect exhibited by the *nup53Δnup59Δ* strain (NP53/NP59-2.1) was clearly demonstrated when these cells were grown competitively with a wild type strain (*Figure 3-2C*). While both strains were seeded in approximately equal amounts, the percentage of NP53/NP59-2.1 cells rapidly decreased with time (*Figure 3-2C*).

Figure 3-2. **Panel A**: *Subcellular distribution of Nup53p and Nup59p.* Yeast strains (NP53PA and NP59pPA) synthesizing protein A-tagged Nup53p (Nup53-pA) or Nup59p (Nup59-pA) were fixed, permeabilized and probed with rabbit anti-mouse IgG followed by donkey anti-rabbit IgG conjugated to the Cy3. The position of the nuclear DNA is visualized by DAPI staining. In each case a punctate perinuclear pattern typical of nuclear pore labeling is observed. Bar, 5 µm. **Panel B**: *Analysis of the genetic interactions between* NUP53, NUP59, *and the genes encoding several abundant nucleoporins.* Haploid strains containing double disruptions of *nup53Δ* and *nup59Δ* and paired combinations of each with *nup170Δ*, *nup188Δ*, *nup157Δ* and *pom152Δ* were constructed and their viability assessed at 30°C. A diagram summarizing the results of these experiments is shown in panel **B**. Solid lines connecting pairs of genes denotes a lethal combination of their null mutations. The gray dashed lines connect pairs of genes which have previously been shown to interact genetically (Aitchison et al., 1995; Nehrbass et al., 1996). The double null combination of *nup53Δnup59Δ* is viable. Shown in **panel C** are the results of a competitive growth assay examining the growth of the *nup53Δnup59Δ* strain (NP53/NP59-2.1) relative to a wild-type haploid strain. YPD cultures were seeded at 0.1 OD_{600} per ml with ~60% NP53/NP59-2.1 and ~40% wild-type cells (t=0). At various times thereafter, the percentage of each strain in the culture was determined and is displayed graphically.


A
Nup53-pA
Nup59-pA
Cy3
DAPI
B
NUP188
NUP157
NUP53
NUP59
POM152
NUP170
C
100
75
50
25
0
Percent
0
5
10
15
20
25
Time (hrs)
□ nup53Δnup59Δ
● Wild-type

3.3 Isolation of an NPC Subcomplex Containing Nup53p, Nup59p, Nup170p, and the Karyopherin Kap121p.

The physical and functional basis for the multiple genetic interactions outlined in *Figure 3-2B* remains largely undefined. To address this question we used C-terminal, protein A-tagged (pA) chimeras of several of these nucleoporins to examine their interactions with one another and other nucleoporins. Here we will focus primarily on experiments conducted with Nup53-pA. On the basis of its localization to the NPC and its ability to complement *nup53Δnup170Δ* and *nup53Δnup188Δ* mutant strains, we concluded that the Nup53-pA chimera functionally replaced wild type Nup53p. Cells expressing Nup53-pA (NP53PA) were lysed under non denaturing conditions. Released Nup53-pA (>95% of the cellular pool) was affinity purified on IgG-Sepharose beads and the bound material analyzed by SDS-PAGE. Five polypeptides with apparent molecular masses of approximately 170 kD, 115 kD, 70 kD, and a closely spaced doublet at 60 kD co-eluted with Nup53-pA (*Figure 3-3*). These species were not present in eluates derived from experiments conducted with a strain lacking the Nup53-pA fusion, or a strain expressing a protein A-tagged nucleolar protein, Ugb-pA (*Figure 3-3*; Tatiana Iouk and Richard Wozniak, personal communication). Both the 170 kD and 115 kD species were consistently found in a near 1:1 stoichiometry with Nup53-pA both when detected by silver staining (*Figure 3-3*) or Coomassie blue staining. The presence of the 70 kD and 60 kD species, however, was more variable suggesting that they are less tightly associated with the Nup53-pA complex.

The molecular masses of two polypeptides in the Nup53p-containing complex suggested that they may be Nup170p and Nup59p. This was in fact established by Western blot analysis performed with anti-Nup170p and anti-Nup59p antibodies (*Figure 3-3*). The 115 kD species was identified by direct microsequencing of proteolytic fragments as the β-karyopherin transport factor Kap121p/Pse1p (YMR308c; Chow et al., 1992; Rout et al., 1997). This was further confirmed by the binding of anti-Kap121p antibodies to the 115 kD protein (*Figure 3-3*). Kap121p is one of two highly similar yeast proteins, the other being Kap123p, that are structurally related to Kap95p and as many as eleven other β-type karyopherin proteins found in yeast. Because of the structural similarity between Kap121p and Kap123p, we tested for the presence of Kap123p in the Nup53p-containing complex by Western blot analysis. No Kap123p was detected in this fraction. In addition, we probed the Nup53p-containing complex with a battery of antibodies that bind to multiple GLFG and FXFG repeat-containing nucleoporins including Nup1p, which has previously been reported to bind Nup170p *in vitro* (Kenna et al., 1996), Nup116p, Nup100p, Nup57p, Nup49p, and the N-terminus of Nup145p. None of these repeat-containing nucleoporins were detected. Similar analysis also failed to detect Nup188p, Nic96p, and Nup157p. These data further support the conclusion that Nup53p specifically interacts with Nup170p, Nup59p, and Kap121p.

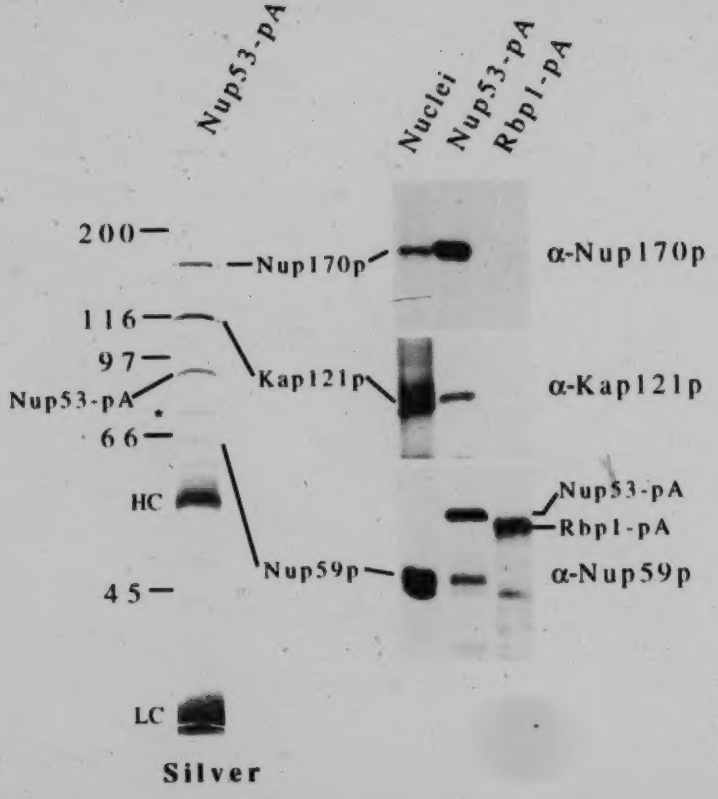


Figure 3-3. Affinity purification of a Nup53-pA containing NPC subcomplex. Lysates derived from cells synthesizing Nup53-pA (NP53PA) or, as a control, a protein A-tagged nucleolar protein, Rbp1-pA were applied to an IgG Sepharose column. Bound material was eluted and the released polypeptides were analyzed by SDS-PAGE. The polypeptide profile of the Nup53-pA eluate was visualized by **silver** staining. The positions of the Nup53-pA and Rbp1-pA fusions are marked. Contaminating heavy (HC) and light chain (LC) IgG fragments are indicated. Four polypeptides were visible that copurified specifically with Nup53-pA. Western blots were performed on polypeptides present in a yeast nuclei fraction, the Nup53-pA eluate, and the Rbp1-pA eluate. These fractions were probed with either anti-Nup170p (**α-Nup170p**), anti-Nup59p (**α-Nup59p**), or anti-Kap121p (**α-Kap121p**) antibodies. Relevant regions of the autoradiograms are shown on the right. Three of four species in the Nup53-pA eluate were identified as Nup170p, Kap121p, and Nup59p. Note, the species comigrating with Nup59p is specifically recognized by the anti-Nup59p antibodies. Additional species observed with the Nup59p antibodies in the Nup53-pA and Rbp1-pA eluates are derived from the protein A fusions. The fourth protein, marked by a single asterisk, was variably present in this fraction and was identified as Ssa1/Ssa2 by direct protein sequencing. The molecular mass markers expressed in kilodaltons are shown on the left.

3.4 *In Vitro* Analysis of the Interactions Between Components of the Nup53p-Containing Complex

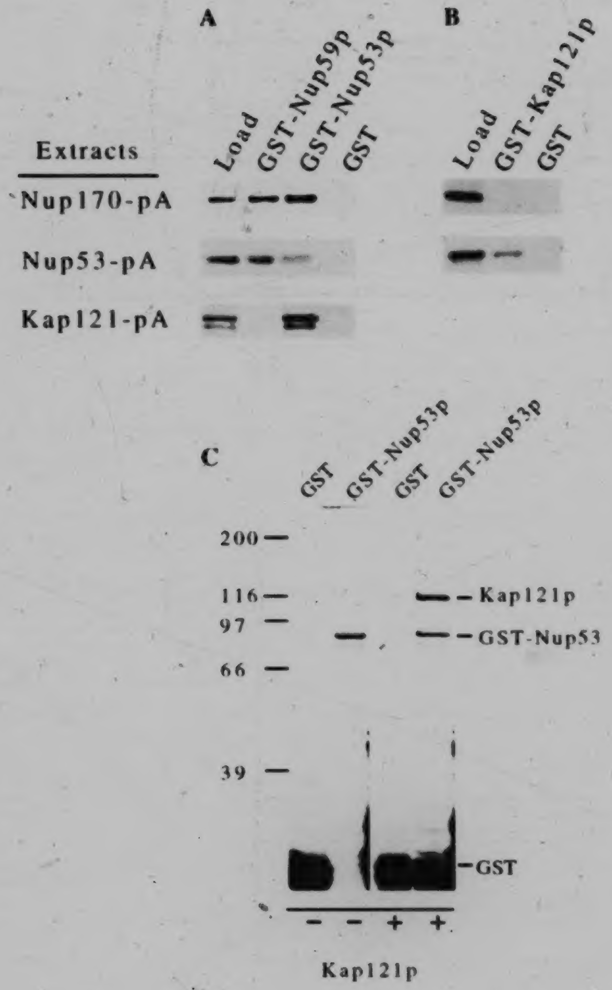
We further investigated the interactions between the identified members of the Nup53p-containing complex using *in vitro* binding analysis. The ORFs encoding three members of this complex, Nup53p, Nup59p, and Kap121p were fused to the 3' end of the glutathione-S-transferase (GST) ORF and expressed in *E.coli*. Each of the resulting GST chimeras were purified on glutathione (GT)-Sepharose beads. The immobilized fusions were tested for their ability to bind protein A chimeras of Nup170p and Nup53p extracted from yeast nuclear envelopes (NEs) and cytosolic Kap121p. For these experiments, NEs were isolated from strains synthesizing either Nup53-pA or Nup170-pA. These NEs were extracted with high salt (1M NaCl) and nonionic detergent to disrupt the NPCs and release the protein A fusions. The salt concentration of the extracts was then adjusted to 150 mM NaCl and the extracts allowed to bind to the immobilized GST fusions. For Kap121-pA, cytosolic fractions derived from cells expressing this fusion were diluted two-fold and used directly for binding. As shown in *Figure 3-4*, the Nup170-pA fusion specifically bound to GST-Nup53p and GST-Nup59p (panel A) but it failed to bind GST-Kap121p (panel B) or the GST alone control. Nup53-pA did bind to GST-Kap121p, GST-Nup59p, and only weakly to the GST form of itself, but not to GST alone. The binding of Kap121p to Nup53p was also evident in experiments where Kap121-pA was incubated with GST-Nup53p (*Figure 3-4A*). However, Kap121-pA did not bind to GST alone and only weakly to GST-Nup59p. Our interpretation of these results is that both Nup53p and Nup59p interact with Nup170p and with each other. We also conclude that Nup53p acts as the major docking site for Kap121p in the Nup53p-containing complex.

The direct interaction of Nup53p and Kap121p was further evaluated using recombinant proteins synthesized in *E.coli*. In these experiments GST-Nup53p and GST alone were immobilized on GT-Sepharose beads and then allowed to bind purified, recombinant Kap121p. Inspection of the bound fraction revealed that Kap121p specifically bound to GST-Nup53p (*Figure 3-4C*), demonstrating that Kap121p binds directly to Nup53p.

3.5 The Interaction of Nup53p with Kap121p is Specific for This β-karyopherin

We next examined whether Nup53p's ability to bind Kap121p was specific for this β-karyopherin. To address this question we used two experimental approaches. First, GST-Nup53p was immobilized on beads and incubated with total yeast cytosol isolated from a Kap121-pA expressing strain (PSE1-A) (*Figure 3-5A*). In addition to Kap121-pA, this cytosolic fraction is likely to contain various known and potential β-karyopherins (Aitchison et al., 1996; Pemberton et al., 1997; Rosenblum et al., 1997; Rout et al., 1997; Schlenstedt et al., 1997; Senger et al., 1998; Hood and Silver, 1998; Lee et al., 1998; Ferrigno et al., 1998; Albertini et al., 1998; Pemberton et al., 1999; Morehouse et al., 1999; Titov and Blobel, 1999). All of the potential yeast β-karyopherins have a predicted molecular mass of between 95-142 kD, similar to wild type Kap121p (see Wozniak et al., 1998). By using cytosol containing the Kap121-pA chimera (~146 kD), the binding of other β-karyopherins to GST-Nup53p could be visualized by SDS-PAGE as additional bands and distinguished from that of the more slowly migrating Kap121-pA. An analysis of the eluates of the GST-Nup53p and GST alone beads are shown in *Figure 3-5A*. A protein of the predicted size of Kap121-pA is clearly visible in the GST-Nup53p eluate. Its identity was confirmed by Western blotting

Figure 3-4. Analysis of the protein-protein interactions between members of the Nup53p-containing NPC subcomplex. Glutathione-S-transferase either alone (GST) or fused to Nup53p (GST-Nup53p), Nup59p (GST-Nup59p), or Kap121p (GST-Kap121p) was synthesized in E.coli and purified on GT-Sepharose beads. These bead were then incubated with extracts derived from yeast NEs containing either Nup53-pA or Nup170-pA or with yeast cytosol containing Kap121-pA. After washing, polypeptides bound to the beads were eluted, separated by SDS-PAGE and analyzed by Western blotting. The protein A fusions were detected with HRP-conjugated donkey anti-rabbit IgG and ECL. The relevant regions of the autoradiograms are shown. The lane marked 'Load' is derived from an aliquot of the protein A chimeric extract used. The results of two separate experiments are shown examining the binding of Nup53-pA, Nup170-pA, and Kap121-pA to GST-Nup53p, GST-Nup59p, and GST alone (**panel A**) or of Nup53-pA and Nup170-pA to GST-Kap121p and GST alone (**panel B**). The ability of Nup53p to directly bind Kap121p was further tested using recombinant proteins. In **panel C**, GST-Nup53p and GST alone were separately bound to GT-Sepharose beads and then incubated with buffer alone (-) or purified, recombinant Kap121p (+). After washing, bound polypeptides were eluted, resolved by SDS-PAGE and detected by silver staining. The positions of Kap121p, GST-Nup53p, and GST are indicated. Molecular mass markers in kilodaltons are indicated on the left.


A
Extracts
Load
GST-Nup59p
GST-Nup53p
GST
Nup170-pA
Nup53-pA
Kap121-pA
B
Load
GST-Kap121p
GST
C
GST
GST-Nup53p
GST
GST-Nup53p
200
116
97
66
39
Kap121p
GST-Nup53
GST
−
−
+
+
Kap121p

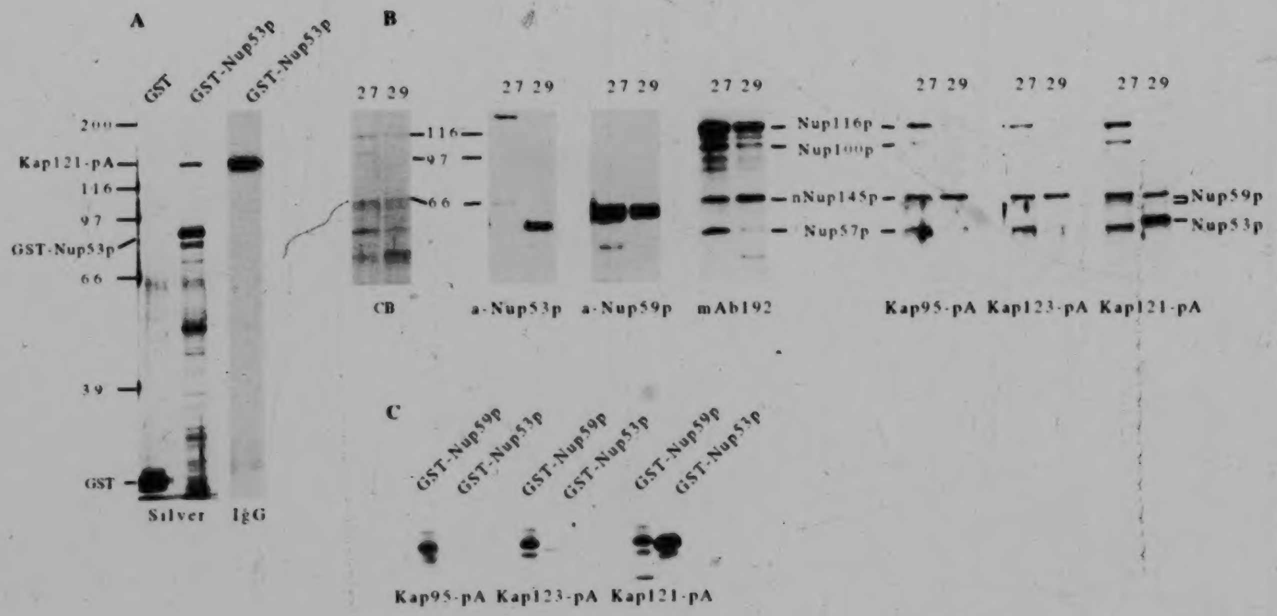
(*Figure 3-5A*). No other polypeptides in the molecular mass range of ~95-142 kD were detected in the GST-Nup53p eluate suggesting that, under these conditions, Kap121p is the only β-karyopherin which binds to Nup53p. Consistent with these observations, Western blots performed with antibodies directed against Kap123p and Kap104p failed to detect these proteins in this fraction.

The specificity of the interactions between Nup53p and Kap121p was further evaluated by blot overlay assays similar to those previously used to identify interactions between several other β-type karyopherins and the GLFG and FXFG repeat-containing nucleoporins (Radu et al., 1995a; Aitchison et al., 1996; Rout et al., 1997; also see Introduction). For these experiments, cytosol from yeast strains synthesizing Kap121-pA, Kap123-pA, and Kap95-pA (Rout et al., 1997) were used to probe yeast nucleoporins present within selected fractions derived from the SDS-hydroxylapatite chromatography of an enriched preparation of yeast NPCs (Wozniak et al., 1994). These fractions contain Nup53p, Nup59p, and several of the repeat-containing nucleoporins including Nup116p, Nup100p, Nup57p, and an N-terminal 65 kD fragment of Nup145p (*Figure 3-5B*). Each of these proteins was identified by Western blotting (*Figure 3-5B*) and, for Nup116p and Nup100p, by direct protein sequencing (Richard Wozniak, unpublished data). While each of the karyopherins bound to the repeat-containing nucleoporins Nup116p, Nup100p, Nup57p and the N-terminus of Nup145p, only Kap121-pA bound to Nup53p (*Figure 3-5B*; Kap121-pA). Strikingly, Kap95-pA and Kap123-pA showed only negligible binding to Nup53p. These results suggest that Kap121p binds specifically to Nup53p as well as to a subset of repeat-containing nucleoporins. The binding of Kap95-pA and Kap123-pA to the repeat-containing nucleoporins is consistent with previous observations (Iovine et al., 1995; Aitchison et al., 1996; Rout et al., 1997; Iovine and Wente, 1997). It is interesting to note that Nup59p (visible as a doublet migrating at approximately 64 kD; *Figure 3-5B*) also bound to each of the karyopherins but lacked the specificity for Kap121p exhibited by Nup53p. These results were further confirmed by performing similar overlay experiments on recombinant GST-Nup53p and GST-Nup59p. As shown in *Figure 3-5C*, Kap121-pA binds strongly to GST-Nup53p while all of the karyopherins tested bound with relatively equal strength to GST-Nup59p.

3.6 RanGTP Releases Kap121p From Nup53p

Rexach and Blobel (1995) have previously shown that an *in vitro* assembled complex of Kap95p/Kap60p (yeast karyopherins β/α) and Nup1p can be disassembled by the GTP-bound form of Ran. We have tested the ability of yeast Ran (Gsp1p) in both the GTP and GDP bound forms to release Kap121p from Nup53p. For these experiments, the GST-Nup53p/Kap121-pA complex was assembled on GT-Sepharose beads as described in the previous section. The complex was then treated with Ran preloaded with GDP, GTP, or the nonhydrolyzable GTP analog GTP-γ-S (*Figure 3-6A*). Kap121-pA was specifically released from GST-Nup53p by RanGTP. GTP hydrolysis did not appear to be required as release was also observed with RanGTP-γ-S. No release was observed with GTP alone and only trace amounts were released with RanGDP (*Figure 3-6A*). These same nucleotide-specific Ran effects are observed on a recombinant Nup53p/Kap121p complex. In addition, we have conducted similar experiments on the isolated Nup53p-containing complex (*Figure 3-6B*). When the complex, bound to IgG-Sepharose, was incubated with RanGTP, Kap121p was also released. After treatment of

Figure 3-5. Nup53p specifically binds Kap121p. Panel A: Recombinant GST-Nup53p and GST alone were bound to GT-Sepharose beads. Each was then incubated with total cytosol isolated from a strain synthesizing Kap121-pA (PSE1-A). Bound polypeptides were resolved by SDS-PAGE and **silver** stained. The positions of Kap121-pA, GST-Nup53p, and GST are indicated. Kap121-pA binds specifically to GST-Nup53p. Its identity was confirmed by Western blotting with nonspecific **IgG** antibodies and visualized with an HRP-conjugated secondary antibody and ECL. No other protein species with an apparent molecular mass greater than GST-Nup53p are detectable. Panel B: Polypeptides in an enriched fraction of yeast NPCs were separated by SDS-hydroxylapatite chromatography. Proteins in two elution fractions, 27 and 29, were separated by SDS-PAGE and either stained with Coomassie blue (**CB**) or transferred to nitrocellulose. Nup53p (**a-Nup53p**), Nup59p (**a-Nup59p**), and several repeat-containing nucleoporins including Nup116p, Nup100p, Nup57p, and the N-terminus of Nup145p (nNup145p) (**mAb192**) were detected in these fractions by Western blotting with the indicated antibody. The positions of these nucleoporins are indicated. Overlay assays were performed on the same fractions (panel B) and recombinant GST-Nup53p and GST-Nup59p (panel C) with total cytosol derived from yeast strains synthesizing **Kap95-pA**, **Kap123-pA**, and **Kap121-pA**. Binding of these fusions to the nucleoporins was detected with HRP conjugated donkey anti-rabbit IgG and ECL. Molecular mass markers in kilodaltons are indicated.


A
GST
GST-Nup53p
GST-Nup53p
200
Kap121-pA
116
97
GST-Nup53p
66
39
GST
Silver
IgG
B
27 29
116
97
66
CB
27 29
a-Nup53p
27 29
a-Nup59p
27 29
mAb192
Nup116p
Nup100p
nNup145p
Nup57p
27 29
Kap95-pA
27 29
Kap123-pA
27 29
Kap121-pA
Nup59p
Nup53p
C
GST-Nup59p
GST-Nup53p
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Kap95-pA
Kap123-pA
Kap121-pA

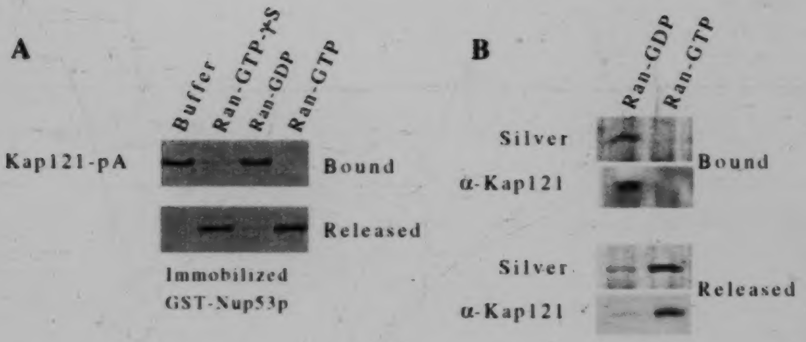

A
Buffer
Ran-GTP-γ-S
Ran-GDP
Ran-GTP
Kap121-pA
Bound
Released
Immobilized
GST-Nup53p
B
Ran-GDP
Ran-GTP
Silver
α-Kap121
Bound
Silver
α-Kap121
Released

Figure 3-6. RanGTP induces the release of Kap121p from Nup53p. **Panel A**: A Kap121-pA/GST-Nup53p complex was formed *in vitro* by incubating yeast cytosol containing Kap121-pA with GST-Nup53p immobilized on GT-Sepharose beads (see *Figure 3-5A*). This complex was then incubated for 30 minutes at room temperature with buffer alone or Ran preloaded with GTP, GDP, or GTP-γ-S. Kap121-pA that was **released** from the column and that which remained **bound** was separated by SDS-PAGE and detected by Western blotting using donkey anti-rabbit conjugated to HRP and ECL. Shown are the relevant regions of the resulting autoradiogram. **Panel B**: The ability of Ran-GTP to release Kap121p from the isolated Nup53-pA complex (see *Figure 3-3*) was examined. Equal amounts of the complex were incubated with RanGTP or RanGDP. Kap121p remaining **bound** to the complex and in the **released** fractions was detected by silver staining (**silver**) and Western blot analysis using anti-Kap121p antibodies (**α-Kap121**). Relevant regions of the gel and autoradiogram are shown.

the complex with RanGDP, however, the majority of Kap121p remained bound. Thus, the mechanism of Kap121p release from Nup53p appears to be similar to that previously observed for the Kap95p/Nup1p complex.

3.7 Steady-State Levels of Kap121p at the NE are Decreased in *nup53Δnup59Δ* Strains

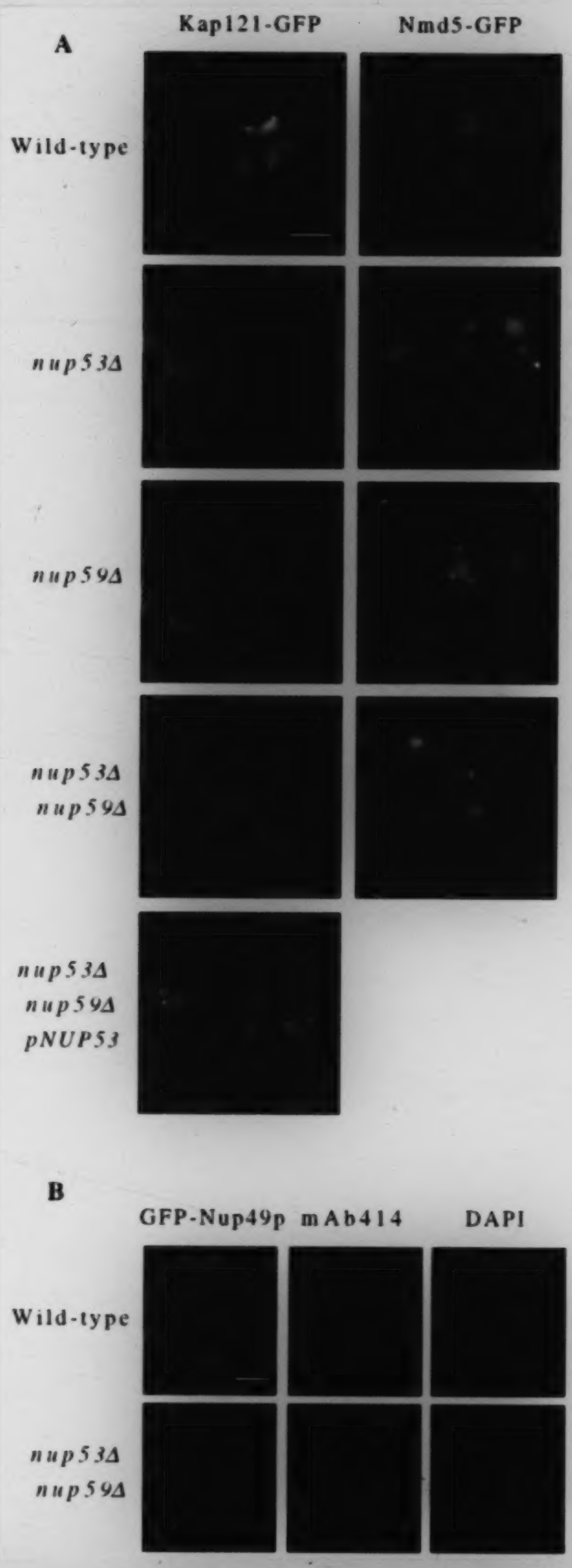
As indicated above, *nup53Δ* and *nup59Δ* haploid strains are viable, as is the slower growing *nup53Δnup59Δ* haploid strain. We have introduced a plasmid-born copy of *KAP121* tagged at its 3' end with the coding region of the green fluorescent protein (*KAP121-GFP*; Seedorf and Silver, 1997) into each of these strains to investigate the effects of removing these nucleoporins on the subcellular distribution of Kap121p. It has been previously shown, by fluorescence microscopy, that in wild type cells Kap121-GFP is concentrated at the NE and diffusely distributed throughout the cytoplasm and the nucleoplasm (Seedorf and Silver, 1997). We observed a similar pattern in DF5 strains (*Figure 3-7*, Wild type) and in the *nup59Δ* strain (*Figure 3-7*). However, inspection of the *nup53Δ* strain revealed that the concentration of Kap121-GFP at the NE was visibly decreased relative to the wild type strain. This reduction in signal was further exaggerated in the *nup53Δnup59Δ* strain where the perinuclear Kap121-GFP signal was greatly reduced or not visible (*Figure 3-7*). In this strain, the Kap121-GFP signal was diffusely distributed throughout the cell. These changes do not appear to be due to massive alterations in the structure of the NPC or NE. No changes were observed in the distribution of GFP-Nup49p or the mAb414-reactive FXFG nucleoporins (*Figure 3-7*) in the *nup53Δnup59Δ* strain. Introduction of a plasmid-born copy of *NUP53* into the *nup53Δnup59Δ* strain restored the perinuclear Kap121-GFP signal (*Figure 3-7*). Thus we conclude that Nup53p, as well as Nup59p, can function as binding sites for Kap121p *in vitro*.

We have performed similar experiments to examine the effects of these various mutants on the cellular distribution of another member of the β-karyopherin family, Nmd5p (Görlich et al., 1997; Rosanna Baker and John Aitchison, unpublished data). As for *KAP121*, *NMD5* was tagged by the addition of *GFP* to the 3' end of the its ORF and the chimera was expressed in the indicated strains. The subcellular distribution of Nmd5-GFP in wild type cells is similar to that observed with Kap121-GFP (R. Baker and J. Aitchison, unpublished data; *Figure 3-7*). However, unlike Kap121-GFP, we observe no changes in the distribution of Nmd5-GFP in any of the *nup53Δ* or *nup59Δ* strains.

3.8 Mutations in *NUP53* Inhibit Kap121p-Mediated Import

Substrates that are imported into the nucleus by Kap121p in wild type cells remain to be identified. However, in strains lacking Kap123p, the import of a Kap123p substrate, the ribosomal protein L25, can be rescued by Kap121p (Rout et al., 1997). We have used this as a model system to examine the effects of *nup53Δ* and *nup59Δ* null mutations on Kap121p-mediated import. For these experiments, *nup53Δkap123Δ* (NP53/KP123) and *nup59Δkap123Δ* (NP59/KP123) haploid strains expressing a reporter gene encoding the L25 NLS linked to β-galactosidase (L25 NLS-β-gal) were isolated. The nuclear import of the L25 NLS-β-gal reporter protein was examined in these strains, as well as in wild type, *nup53Δ*, and *kap123Δ* haploid strains, by indirect immunofluorescence microscopy. In wild type and *nup53Δ* (*Figure 3-8*) strains, the L25

Figure 3-7. Cellular distribution of Kap121-GFP and Nmd5-GFP in nup53Δ and nup59Δ null mutants. **Panel A**: A plasmid-born copy of the *KAP121-GFP* or *NMD5-GFP* chimeric gene was introduced into four haploid yeast strains: Wild type DF5, *nup53Δ* (NP53-B1), *nup59Δ* (NP59-23), *nup53Δnup59Δ* (NP53/NP59-2.1), and, for *Kap121-GFP*, the *nup53Δnup59Δ* strain containing a plasmid-born copy of *NUP53* (pRS315-NUP53). In each case, cells were grown to mid-log phase and examined directly by fluorescent microscopy. **Panel B**: The distribution of GFP-Nup49p was examined in wild-type DF5 and *nup53Δnup59Δ* strains. Immunofluorescence microscopy was also performed on these strains using mAb414 to detect the localization of FXFG-containing nucleoporins. The position of the nuclear DNA is visualized by DAPI staining. Bar, 5 μm.


A
Kap121-GFP
Nmd5-GFP
Wild-type
nup53Δ
nup59Δ
nup53Δ nup59Δ
nup53Δ nup59Δ pNUP53
B
GFP-Nup49p
mAb414
DAPI
Wild-type
nup53Δ nup59Δ

NLS-β-gal reporter is localized exclusively to the nucleus. However, consistent with previous observations (Rout et al., 1997; Schlenstedt et al., 1997), in the *kap123Δ* strain we observe, in addition to nuclear staining, a distinct increase in the amount of the L25 NLS-β-gal reporter protein present in the cytosol. Strikingly, in the *nup53Δkap123Δ* strain the L25 NLS-β-gal reporter protein is dramatically mislocalized to the cytosol (*Figure 3-8*). This effect is not observed in *nup59Δkap123Δ* strains which exhibits a staining pattern similar to that seen in the *kap123Δ* strain. These results further suggest that Nup53p plays a direct role in Kap121p-mediated import.

3.9 Components of the Nup53p-Containing Complex are Present on Both the Cytoplasmic and Nucleoplasmic Faces of the NPC

We have attempted to localize the Nup53p-containing complex by examining the distribution of Nup53p, Nup59p, and Nup170p within the NPC using a preembedding, immunolabeling technique on isolated NEs. This technique allows us to approximate the position of nucleoporins relative to the central plane of the NPC. It has previously been used to establish the symmetrical distribution of Nup188p on both faces of the NPC core (Nehrbass et al., 1996) and to localize Nup159p to the cytoplasmic face of the NPC (Kraemer et al., 1995). NEs isolated from strains synthesizing protein A-tagged chimeras of either Nup53p, Nup59p, Nup170p or Nup157p were probed with purified rabbit antibodies and secondary antibodies coupled to gold particles. Samples were then embedded, sectioned and viewed by electron microscopy. In all cases, gold particles were visible on both the cytoplasmic and nucleoplasmic faces of the NPC (*Figure 3-9*). In sections that were cut perpendicular to the plane of the NE and through the NPC, we counted the number of gold particles and measured their distance from the midplane of the NPC. As shown in *Figure 3-9*, for each of these nucleoporins, gold particles were distributed in nearly equal amounts on both the nucleoplasmic and cytoplasmic faces of the NPC. In all cases, the majority of the gold particles lie within 30 nm of the midplane of the NPC, with a peak distribution occurring between 15-25 nm. By comparison, in similar experiments performed with Nup188p (Nehrbass et al., 1996) and Nup159p (Kraemer et al., 1995), the greatest concentration of particles was observed at distinctly different distances of between 5-15 nm and 30-50 nm, respectively, from the midplane of the NPC. The similar labeling patterns observed with Nup53p, Nup59p, and Nup170p are consistent with their physical association within an NPC substructure.

3.10 Nup53p is Phosphorylated During Mitosis

As indicated above (*Figure 3-1*), a comparison of yeast Nup53p with protein sequence data bases identified a potential *Xenopus laevis* homologue termed MP44. MP44 was identified by Stukenberg et al. (1997) on the basis of its ability to act as a substrate for mitosis-specific kinases present in *Xenopus* egg extracts. However, the location and function of MP44 was not addressed. To determine whether Nup53p and Nup59p are also mitotic phosphoproteins, we have introduced plasmid-born copies of the *NUP53-pA* and *NUP59-pA* genes into the yeast cell-cycle mutant cdc15-2 (Hartwell et al., 1971; Pringle and Hartwell, 1981). In this temperature-sensitive strain, a shift to the non permissive temperature (37°C) arrests cells in late mitosis and maintains the M-phase specific kinase, $p34^{cdc2}$, in an active state (Surana et al., 1993). Haploid cdc15-2 cells synthesizing either Nup53-pA (cdc15-2-53) or Nup59-pA (cdc15-2-59) were grown at

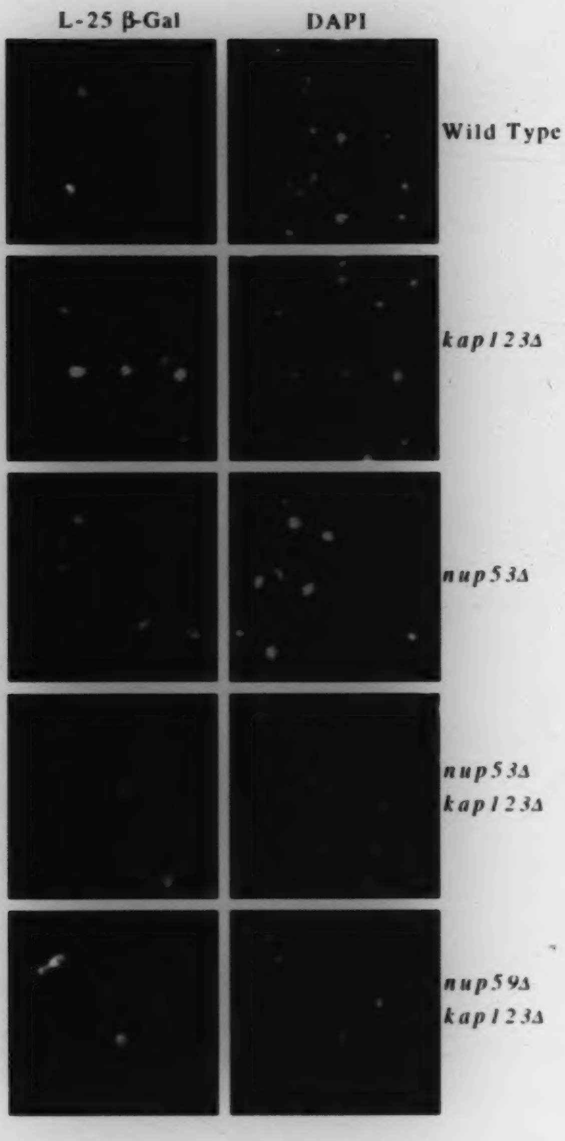

L-25 β-Gal
DAPI
Wild Type
kap123Δ
nup53Δ
nup53Δ
kap123Δ
nup59Δ
kap123Δ

Figure 3-8. Deletion of the NUP53 gene inhibits Kap121p-mediated import. A plasmid-born copy of an L25 NLS-β-galactosidase chimeric gene was introduced into four haploid yeast strains: wild type DF5, *kap123Δ* (123Δ-14-1), *nup53Δ* (NP53), *nup53Δkap123Δ* (NP53/KP123), and *nup59Δkap123Δ* (NP59/KP123). Cells were grown to mid-log phase, fixed, permeabilized and probed with mouse monoclonal anti-β-galactosidase antibody. Binding was detected with goat anti-mouse IgG conjugated to rhodamine. The position of the nuclear DNA is visualized by DAPI staining. Bar, 5 μm.

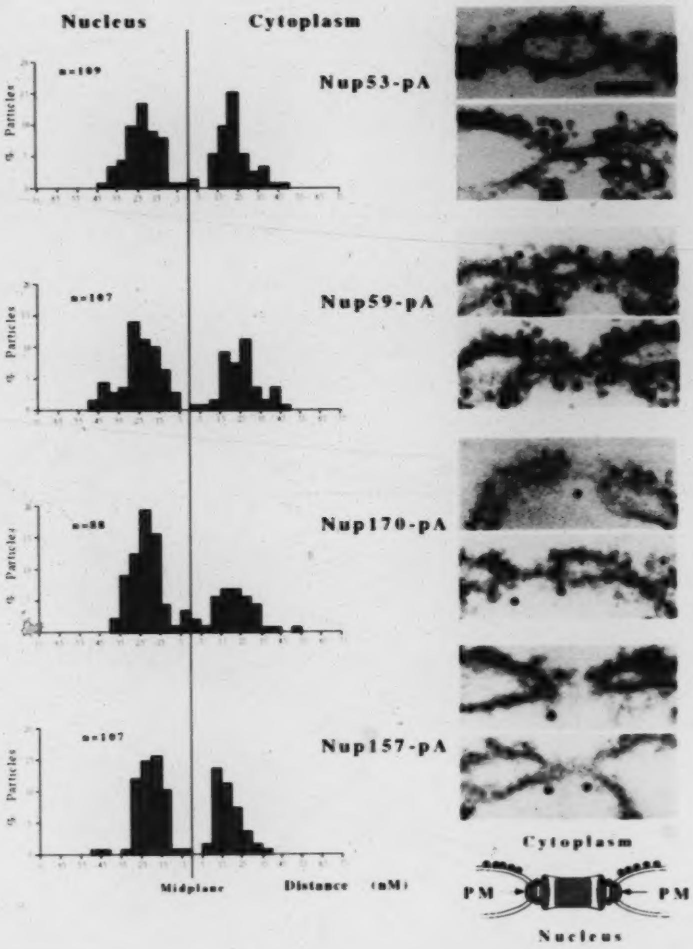
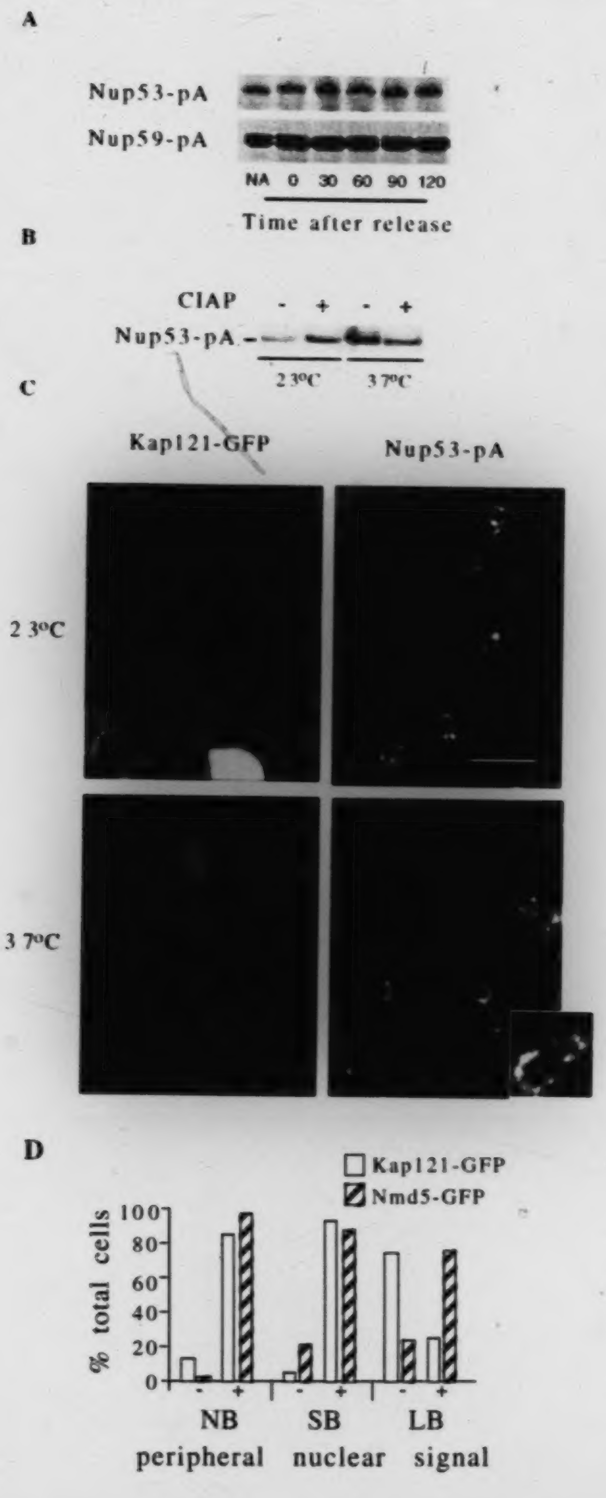


Figure 3-9. Nup53p, Nup59p, Nup170p, and Nup157p are symmetrically distributed on both faces of the NPC. The localization of protein A-tagged Nup53p, Nup59p, Nup170p, and Nup157p was examined in NEs isolated from four separate yeast strains. The chimeras were visualized by the binding of rabbit IgG and either 5 nm (Nup53-pA) or 10 nm (Nup59-pA, Nup157-pA, and Nup170-pA) gold-labeled secondary antibodies. On the right are shown two representative micrographs of each labeled nucleoporin. In each case, the NEs are oriented as shown in the model below the micrographs (PM, pore membrane). All four of these nucleoporins are accessible on both faces of the NPC in multiple copies per pore. Bar, 0.1 µm. On the left are shown the histogram quantification of the distribution of gold particles (n= total number counted) as measured from the midplane of the associated NPC to the center of the gold particle. Positive distances are assigned to particles on the cytoplasmic side of the NPC and negative distances to those on the nucleoplasmic side.

the permissive temperature (23°C), shifted to the non permissive temperature (37°C) for 3.5 h, and then allowed to recover at 23°C. The states of Nup53-pA and Nup59-pA at various time points were analyzed by Western blotting (*Figure 3-10A*). At 23°C Nup53-pA was visible largely as a single band with an apparent molecular mass identical to that observed in asynchronous wild type cells. However in M-phase arrested cultures, we observed a distinct reduction in the mobility of Nup53-pA (*Figure 3-10*; t=0). This mobility shift can be largely eliminated by treatment of the arrested extracts with calf intestinal alkaline phosphatase (*Figure 3-10B*), implying that the mass shift is due to the addition of phosphate. Moreover, the mass shift in Nup53-pA was reversed upon release of the mitotic block induced by shifting the cells to 23°C for 30 min or more (*Figure 3-10A*). In parallel experiments, however, we did not observe alterations in the mobility of the Nup59-pA doublet (*Figure 3-10A*).

The mitosis-specific phosphorylation of Nup53p raises the intriguing possibility that this modification alters its interactions with other nucleoporins and Kap121p. To begin to address this question, we have examined the subcellular distribution of Nup53-pA and Kap121-GFP in *cdc15-2/cdc15-2* homozygous diploid strains (cdc15/cdc15-2B-53 and cdc15/cdc15-2B-121) at permissive and non permissive temperatures. In both asynchronous and M-phase arrested cultures, Nup53-pA is located at the nuclear periphery in a distinct punctate pattern (*Figure 3-10C*), suggesting that phosphorylation does not affect its localization to the NPC. The distribution of Kap121-GFP in cdc15/cdc15-2B-121 cells at the permissive temperature also appears as in wild type cells, exhibiting both diffuse cytoplasmic and nucleoplasmic staining as well as a concentration along the nuclear periphery (*Figure 3-10C*, 23°C). However, in cells arrested in M-phase after shifting to 37°C for 3.5 h (*Figure 3-10C*, 37°C), Kap121-GFP was visible throughout the cytoplasm and the nucleoplasm, but was no longer concentrated at the NE. Similar temperature shifts performed on wild type DF5 cells synthesizing Kap121-GFP had no effect on its subcellular distribution. The mitotic specific redistribution of Kap121-GFP was also examined in an asynchronous culture of DF5 cells. Nonbudded, small budded, and large budded cells were randomly identified and then examined by fluorescent microscopy to evaluate the cellular distribution of Kap121-GFP. We observed that in most of the nonbudded (86%) and small budded (82%) cells Kap121-GFP was visibly concentrated at the NE (*Figure 3-10D*). In contrast, only 12% of large budded cells, the majority of which are in the late M-phase (Byers, 1981), exhibit a NE signal. In most of these cells (88%), Kap121-GFP is not concentrated at the NE and is visible as a diffuse signal throughout the cytoplasm and nucleus, similar to the pattern observed in arrested cdc15-2 strains. By comparison, Nmd5-GFP is concentrated at the NE in the majority of cells regardless of their stage in the cell-cycle (*Figure 3-10D*). These observations suggest that the localization of Kap121-GFP to the NE, but not Nup53-pA or Nmd5-GFP, is modulated during mitosis.

Figure 3-10 **Panels A** and **B**: *Mitosis-specific phosphorylation of Nup53-pA.* Cdc15-2 cells expressing *NUP53-pA* (cdc15-2-53) and *NUP59-pA* (cdc15-2-59) were grown at the permissive temperature of 23°C to early log phase (non-arrested) before being shifted to 37°C (non-permissive temperature) for 3.5 h. The arrested cultures were then released from M-phase arrest by shifting the culture back to 23°C. Whole cell lysates from non-arrested (NA), cells arrested for 3.5 h (t=0), and cells released from arrest for various times (t=30 to t=120 min) were isolated. Polypeptides in these samples were separated by SDS-PAGE and then analyzed by Western blotting to detect the Nup53-pA or Nup59-pA fusion proteins. As shown in **panel A**, an M-phase specific decrease in the electrophoretic mobility of the Nup53-pA, but not Nup59-pA, was observed in arrested cultures (t=0). This change in mobility was reversed after the cultures were returned to the permissive temperature. Extracts from cdc15-2-53 cells grown at 23°C or arrested for 3.5 h at 37°C were treated with (+) or without (-) calf intestinal alkaline phosphatase (CIAP, **panel B**). The molecular mass of Nup53-pA was then evaluated as in panel A. In panels A and B, relevant regions of the gel and autoradiogram are shown. **Panel C** and **D**: *Alterations in the localization of Kap121-GFP in arrested cdc15/cdc15 and wild-type strains.* The cellular distribution of Kap121-GFP in the diploid strain cdc15/cdc15-2B was examined by fluorescence microscopy at the permissive temperature of 23°C and 3.5 hr after a shift to 37°C to arrest cells in M-phase (panel C). The distribution of Nup53-pA in the cdc15/cdc15-2B-53 strain at the permissive temperature (23°C) and in the arrested cultures (37°C) was examined by immunofluorescence microscopy (panels on the right). Bar, 5 µm. In **panel D** a histogram displays the results of experiments examining the subcellular distribution of Kap121-GFP in an asynchronous culture of wild-type DF5 cells. Approximately 300 nonbudded (NB), small budded (<70% of the diameter of the mother cell; SB), and large budded (>70% of the diameter of the mother cell; LB) cells were identified by phase microscopy and then scored for the presence of a NE-associated Kap121-GFP signal in the fluorescein channel. The relative percentage of cells showing (+) or not showing (-) a distinct perinuclear signal in each group of cells is shown.


A
Nup53-pA
Nup59-pA
NA
0
30
60
90
120
Time after release
B
CIAP
-
+
-
+
Nup53-pA
23°C
37°C
C
Kap121-GFP
Nup53-pA
23°C
37°C
D
Kap121-GFP
Nmd5-GFP
% total cells
100
80
60
40
20
0
NB
SB
LB
peripheral
nuclear
signal

3.11 Discussion

Recent biochemical and structural analysis suggest that the yeast NPC is likely composed of ~30 proteins (Rout et al., 2000). Of these a subset of constituents has been proposed to form the repetitive structures of the NPC core (Aitchison et al., 1996). These proteins likely provide binding sites for the attachment of the cytoplasmic and nucleoplasmic filaments and a framework on which other nucleoporins, including the GLFG and FXFG repeat-containing nucleoporins, are spatially organized and presented to the soluble transport machinery. Such an ordered array of nucleoporins likely plays a fundamental role in vectorial transport through the NPC.

We have identified a subcomplex of the NPC that establishes a link between a putative core component of the NPC and the soluble transport machinery. This subcomplex contains the abundant nucleoporin, Nup170p, two structurally related nucleoporins, Nup53p and Nup59p, and the β-karyopherin Kap121p. On the basis of the near quantitative recovery of the Nup53-pA chimera used to isolate this complex, we conclude that it is largely derived from disassembled NPCs. *In vitro* binding studies suggest that Nup170p and Kap121p do not directly interact but instead are linked through Nup53p. Nup59p is also present in this complex, however in lesser and variable amounts suggesting it is more loosely bound. The conclusion is further supported by *in vitro* binding studies (*Figure 3-4*) and two-hybrid analysis (Sergei Tcheperegine and Richard Wozniak, unpublished data) which show that Nup59p interacts with Nup53p and Nup170p. We have also investigated whether Nup1p is associated with the Nup53p-containing complex. In previous work conducted by Kenna et al. (1996) it was suggested that Nup1p may also interact with Nup170p. However, we failed to detect Nup1p in this isolated complex.

The association of Kap121p with this complex of nucleoporins is most likely mediated by its binding to Nup53p. We have drawn this conclusion on the basis of their ability to specifically interact with one another under various experimental conditions (*Figure 3-5A, 3-5B, 3-5C*). Strikingly, we observe that the interaction between Nup53p and Kap121p is specific for this β-karyopherin. This is underscored by experiments demonstrating that Nup53p only binds Kap121p when presented with a pool of karyopherins present in total yeast cytosol, including a highly similar and 10-fold more abundant karyopherin, Kap123p (Rout et al., 1997; Schlenstedt et al., 1997). Moreover, overlay assays used to examine the ability of different β-karyopherins to bind Nup53p also reflects this specificity (*Figure 3-5*). In addition to these interactions, two other observations support the idea that Nup53p plays an important physiological role in the Kap121p-mediated transport pathway. First *nup53Δ* mutations, either alone or, more strikingly, in combination with *nup59Δ* mutations, alter the nuclear envelope association of Kap121p (*Figure 3-7*). Second, consistent with this phenomenon, the Kap121p-mediated import of an L25 NLS-β-galactosidase reporter is inhibited in the absence of Nup53p (*Figure 3-8*).

It is likely that Nup59p also serves as a binding site for Kap121p as well as other β-karyopherins (*Figure 3-5*). We detect weaker but specific interactions between Nup59p and Kap121p, Kap123p, and Kap95p using overlay assays. These results suggest that both Nup53p and Nup59p function in binding Kap121p at the NPC, although their apparent differences in affinity and specificity for Kap121p suggest they perform separate functions. The elimination of these sites may significantly alter Kap121p-mediated transport, a process which is presumed to be vital as deletion mutants of *KAP121* are lethal (Rout et al., 1997; Seedorf and Silver, 1997). This would explain the growth defects and the competitive disadvantage experienced by the *nup53Δnup59Δ* double null strain relative to wild type strains (*Figure 3-2C*).

The *nup53Δnup59Δ* mutant, however, is viable suggesting that Kap121p can either by-pass these binding sites or utilize alternative pathways through the NPC. Either scenario would suggest that Kap121p can interact with other nucleoporins. Candidate proteins include the GLFG and FXFG repeat-containing nucleoporins. As shown in *Figure 3-5*, Kap121p can bind to, at least, a subset of these proteins including Nup116p, Nup100p, Nup57p and the N-terminal region of Nup145p. These results are not surprising. Various approaches, in both yeast and vertebrates, have established that a number of β-karyopherins, as well as other potential transport factors, will bind to this group of nucleoporins (see Chapter 1). In yeast, this has been shown using a number of experimental approaches including blot overlay, *in vitro* binding, FRET, and two-hybrid assays (Rexach and Blobel, 1995; Iovine et al., 1995; Aitchison et al., 1996; Rout et al., 1997; Pemberton et al., 1997; Rosenblum et al., 1997; Iovine and Wente, 1997; Daemlin and Silver, 2000). It is intriguing to note, however, that individual karyopherins can bind separate but overlapping subsets of the repeat-containing nucleoporins. For example, using overlay assays, both Kap104p and Kap95p bind to Nup116p, but of the two only Kap95p appears to bind Nup1p (Aitchison et al., 1996). Moreover, the relative strength of the interactions between individual karyopherins and nucleoporins also varies (see Aitchison et al., 1996; Rout et al., 1997; Pemberton et al., 1997; Rosenblum et al., 1997).

This selectivity has also been observed in vertebrates using overlay assays (Bonifaci et al., 1997; Yaseen and Blobel, 1997) and by co-immunoprecipitation (Fornerod et al., 1997a; Shah et al., 1998). Using the latter approach, stable associations have been identified between human Nup214p and Crm1p (Fornerod et al., 1997a) and Xenopus Nup153p and karyopherin β implicating these as high affinity binding sites (Shah et al., 1998). These results, like those we have obtained with Nup53p and Kap121p, support the hypothesis that individual or groups of karyopherins may follow separate but overlapping pathways through the NPC. These pathways are likely to contain binding sites that are preferred by individual karyopherins and thus they could create distinct intermediate transport steps. Functionally this could provide a mechanism for improving the efficiency of transport and a means of regulating specific transport pathways at the level of the NPC (see below).

Upon close inspection of the amino-acid sequence of Nup53p and Nup59p we identified sequence elements which suggest that these proteins may be distantly related members of the GLFG and FXFG repeat-containing family of nucleoporins. Both Nup53p and Nup59p contain several dispersed FG repeat sequences (four in Nup53p and six in Nup59p). Moreover, one of these lies within a stretch of amino acid-residues which is highly conserved in all the potential Nup53p homologues (*Figure 3-1B*) suggesting these regions play an important functional role. However, the similarity to the repeat-containing family is largely restricted to these FG residues. Unlike all the other members of the repeat family, neither Nup53p nor Nup59p contains any repetitive GLFG or FXFG consensus sequences. Moreover, they are not recognized by monoclonal (mAb414, mAb192) or polyclonal (anti-GLFG) antibodies which recognize multiple members of this family. Searches of sequence databases also failed to detect any similarities between Nup53p or Nup59p and the GLFG and FXFG repeat-containing proteins. However, the apparent functional similarity between these proteins, i.e. their ability to bind karyopherins, does suggest that they contain similar structural elements. The divergence of the Nup53p sequence from this consensus may in fact be responsible for its specific interaction with Kap121p.

Like several other members of the β-karyopherin family, Kap121p has previously been shown to bind RanGTP (Görlich et al., 1997). We have shown that Kap121p bound to Nup53p in various contexts, both within the isolated NPC subcomplex and using recombinant proteins, can be released by RanGTP, but not by RanGDP. These results are similar to those previously reported for the release of Kap95p from Nup1p (Rexach and Blobel, 1995). On the basis of these data, Rexach and Blobel (1995) proposed that RanGTP plays a direct role in the release of the Kap95p/Kap60p/cNLS cargo from multiple nucleoporin binding sites as it moves through the NPC. Since RanGTP also dissociates the Kap95p/Kap60p/cNLS-cargo complex (Rexach and Blobel, 1995), it has been suggested that this reaction is restricted to the terminal stages of transport on the nucleoplasmic face of the NPC where it would presumably be exposed to nuclear RanGTP (Görlich et al., 1996). Our immunolocalization studies suggest that the Nup53p-containing complex is accessible on both the cytoplasmic and nucleoplasmic faces of the NPC. The RanGTP-dependent release of Kap121p from these sites would thus require local concentrations of RanGTP on both faces of the NPC. Moreover, the symmetrical localization of Nup170p, which has also been implicated as a binding site for Nup1p (Kenna et al. 1996), suggests that Nup1p may also be located on both faces of the NPC. These data are consistent with a model in which RanGTP dissociates karyopherins from nucleoporins at multiple locations within the NPC.

Somewhat surprisingly we observed that Nup53p is phosphorylated during mitosis. Moreover, yeast Nup53p is specifically phosphorylated during M-phase in cycling *Xenopus* egg extracts (M. Marelli, E. Shibuya, and R.Wozniak, unpublished data). Several potential sites for the mitotic kinase $p34^{cdc2}$ exist in Nup53p. Which of these sites are phosphorylated in Nup53p remains to be determined. Nup59p, whose mobility is not altered during mitosis in yeast, also contains several potential phosphorylation sites. Whether these sites are phosphorylated is currently under investigation.

Nup53p is the first yeast nucleoporin shown to be phosphorylated during mitosis. In metazoan cells several NPC proteins, including the pore membrane protein Gp210 and the repeat-containing nucleoporins Nup153p, Nup214p, Nup358p (Favreau et al., 1996), and a 97 kD protein (possibly Nup98; Macaulay et al., 1995) are phosphorylated (or hyperphosphorylated) during mitosis. In these cells it has been largely assumed that the mitotic specific phosphorylation of NPC proteins plays a role in disassembling this structure during nuclear envelope breakdown. However, yeast do not disassemble their nuclear envelopes or NPCs during mitosis (Byers, 1981; Copeland and Snyder, 1993). Consistent with this observation, the subcellular distribution of Nup53p is not changed in M-phase arrested *cdc15-2* strains (*Figure 3-10*). An alternative function for this modification may be to alter Nup53p's association with specific nucleoporins or Kap121p. Intriguingly, the nuclear envelope concentration of Kap121-GFP is reduced in late M-phase in both arrested cdc15/cdc15-2B-121 and wild type cells. One plausible explanation for these data is that steady-state levels of NPC-bound Kap121p are altered either by the inhibition of binding to, or by a more rapid release from, Nup53p. A consequence of this could be the modulation of import (or export) of Kap121p cargo. The functional significance of this will await the identification of Kap121p's repertoire of substrates. One can envision that such an M-phase specific change in nuclear transport could also occur in higher eukaryotes as well, perhaps as a prelude to nuclear envelope breakdown or following its reassembly.

Chapter 4

Examination of the Role of Nup53p in Nucleo-Cytoplasmic Transport and NPC Formation

4.1 Overview

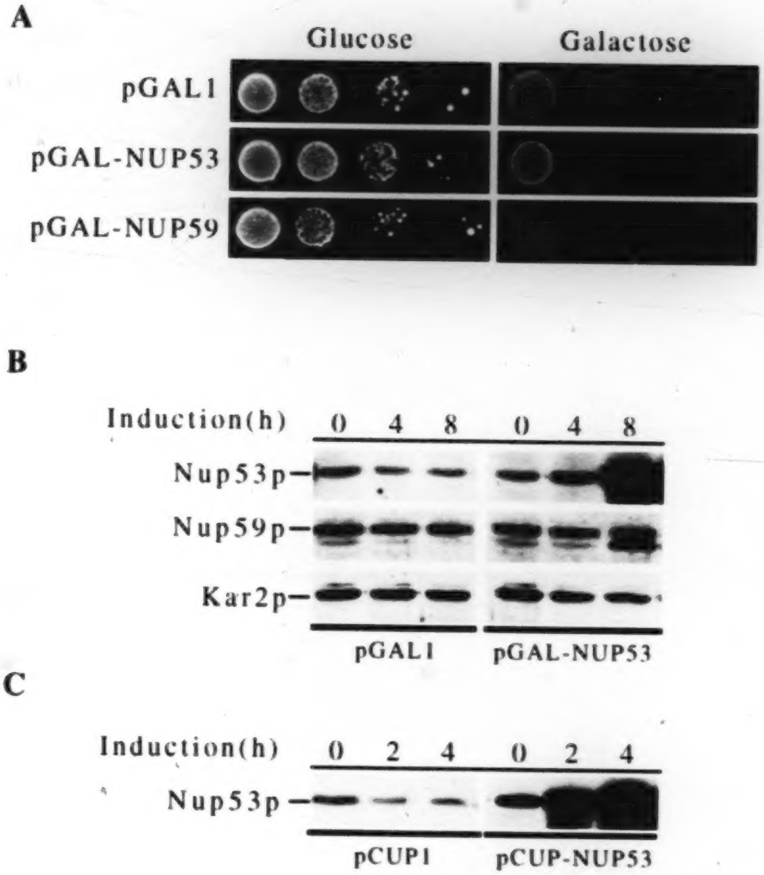
To further examine the role of Nup53p in Kap121p-mediated import, we overexpressed *NUP53* in yeast cells. Increased dosages of Nup53p resulted in the specific inhibition of Kap121p-dependent import of Pho4p, but not of a Kap95p-mediated import cargo. Immunofluorescence microscopy indicated that the excess Nup53p in induced cells accumulated at the nuclear periphery in a continuous smooth pattern distinct from the punctate staining observed at wild type levels of Nup53p. However, the localization of Kap121p was not altered in induced cells. Strikingly, overexpression of *NUP53* induced the accumulation of intranuclear double-membrane stacks that appeared to originate from the inner nuclear membrane. These membranes contained transcisternal pores and the integral pore-membrane proteins Pom152p and Ndc1p, but lacked fully assembled NPCs. The nuclear localization of excess Nup53p and the proliferation of membranes were inhibited in *Kap121-41* and *nup170Δ* mutant cells. Interestingly, Kap121p and Nup170p are required for the efficient assembly of endogenous Nup53p at the NPCs.

4.2 Overproduction of Nup53p Specifically Inhibits Kap121p-Mediated Nuclear Import

We have previously shown that the yeast Nup53p acts as a specific binding site for the karyopherin Kap121p (Marelli et al., 1998). To further characterize Nup53p's role in import, we overexpressed the *NUP53* gene to investigate the effects that increased dosages of this nucleoporin may have on the Kap121p transport pathway. Inducible, high expression levels of this nucleoporin, or a structurally related Nup59p, was achieved by placing the ORF of *NUP53* (or *NUP59*) under the control of either the *GAL1* promoter of pGAL1 (*2µM-TRP1*), or the copper responsive *CUP1* promoter in the plasmids pCUP1 (URA3-*leu2d*) and pRS315-CUP1 (*LEU2*). Each of these plasmids produced elevated levels of Nup53p upon induction in W303 cells. As a consequence, these plasmids were used interchangeably in the experiments described below. In both cases, we observed that cells overexpressing *NUP53* and *NUP59* displayed an attenuation of growth on induction media when compared to cells containing an empty plasmid (*Figure 4-1A*). To examine the relative changes in Nup53p levels in response to induction, Western blot analysis of whole cell lysates prepared from W303 cells containing pGAL-NUP53 (GNP53) showed elevated levels of Nup53p within 4 h which continued to increase through 8 h after induction (*Figure 4-1B*). By comparison, W303 cells containing pCUP-NUP53 (CNP53) responded more rapidly to the induction conditions and a detectable increase in the levels of Nup53p was observed within 2 h, which increased continuously through 4 h after induction (*Figure 4-1C*). CNP53 also displayed somewhat higher basal levels of expression. No changes in the levels of Nup53p were observed in cells containing the corresponding empty plasmids. The levels of other NPC components like Nup59p or an unrelated endoplasmic reticular protein, Kar2p, were not affected in response to the overproduction of Nup53p (*Figure 4-1B*). The relative increase in Nup53p over endogenous levels was difficult to estimate; however a low level of cross reactivity of the Nup59p-reactive antibodies with Nup53p illustrates the relative increase in the dosage of Nup53p when compared to Nup59p (*Figure 4-1B*; α-Nup59).

Our observations that mutations in *NUP53* inhibit Kap121p-mediated import led us to examine the effect of overproducing Nup53p on this import pathway. This was

Figure 4-1. Induction of NUP53 overexpression results in decreased growth rates and increased Nup53p production. **Panel A**: serial dilutions of wild type W303 cells containing a pGAL1, pGAL-NUP53 (GNP53), or pGAL-NUP59 plasmid were spotted onto selective media containing 2% glucose or 2% galactose as the sole carbon source and allowed to grow at 30°C for two days. **Panel B**: Logarithmically growing GNP53, or W303 cells containing pGAL1, were induced by transferring them to galactose containing media for the indicated times. Whole cell lysates prepared from these cultures were analyzed by Western blotting using rabbit polyclonal antibodies directed against Nup53p, Nup59p, and the endoplasmic reticular protein Kar2p, or a monoclonal antibody (mAb118C3) that binds Pom152p. Binding was detected with HRP-conjugated secondary antibodies and ECL. The anti-Nup59p antibody reacts weakly with Nup53p leading to the appearance of a 53 kilodalton species after 8 h of induction. **Panel C**: Wild type W303 cells containing either a pCUP1, or pCUP-NUP53 (CNP53) plasmid were induced with 0.5 mM copper sulfate for the indicated times and analyzed as indicated above using anti-Nup53p antibodies.


A
Glucose
Galactose
pGAL1
pGAL-NUP53
pGAL-NUP59
B
Induction(h)
0 4 8 0 4 8
Nup53p
Nup59p
Kar2p
pGAL1
pGAL-NUP53
C
Induction(h)
0 2 4 0 2 4
Nup53p
pCUP1
pCUP-NUP53

assessed using a reporter protein containing the NLS of the transcription factor Pho4p coupled to GFP (Pho4-GFP; Kaffman et al., 1998b), a substrate dependent on Kap121p for its nuclear localization. For these experiments, CNP53 cells expressing the Pho4-GFP reporter were grown in selection media in the presence or absence of copper ions (*Figure 4-2*). Consistent with previous reports (Kaffman et al., 1998), Pho4-GFP was concentrated in the nucleus in wild type cells containing an empty pCUP1 plasmid under inducing (+Cu2$^+$) and non-inducing (-Cu2$^+$) conditions (*Figure 4-2*). CNP53 cells also showed nuclear accumulation of Pho4-GFP under non-inducing conditions, but induced cultures displayed a marked redistribution of Pho4-GFP to the cytoplasm. In some cells, the Pho4-GFP could also be observed accumulating at the nuclear periphery.

The effect of Nup53p overproduction was specific to the Kap121p-mediated import pathway. Similar experiments were performed on cells in which the Pho4-GFP plasmid was replaced by a plasmid encoding a 'classic' NLS-GFP fusion that is imported into the nucleus by Kap95p (Shulga et al., 1996). In these strains cNLS-GFP localized to the nucleus in both control and Nup53p overproducing strains (*Figure 4-2*).

4.3 Overproduced Nup53p Accumulates Along the Periphery of the Nuclear Envelope

To understand the basis for the specific inhibition of Kap121p mediated import caused by the overproduction of Nup53p, we examined the localization of Nup53p and its effect on the distribution of Kap121p. Cells containing the pCUP-NUP53 overexpression plasmid were induced with copper sulfate and examined by indirect immunofluorescence microscopy using affinity-purified, anti-Nup53p polyclonal antibodies (*Figure 4-3A*).

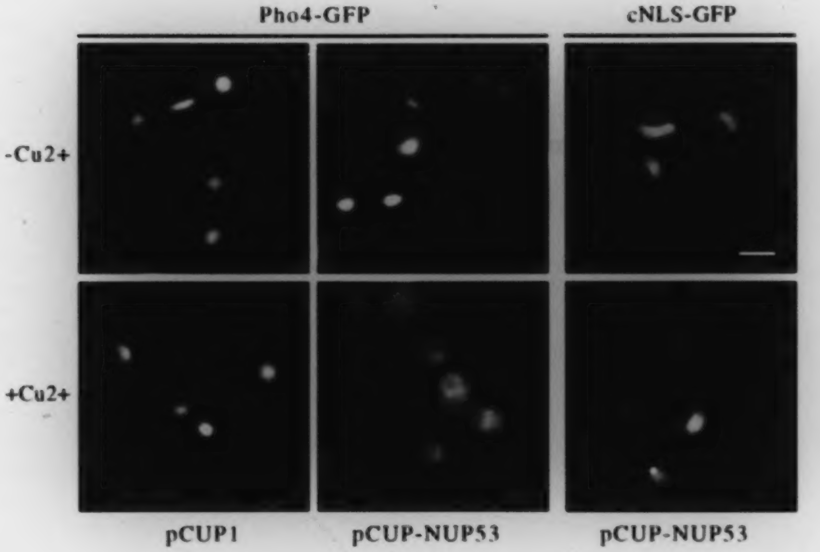

Pho4-GFP
cNLS-GFP
-Cu2+
+Cu2+
pCUP1
pCUP-NUP53
pCUP-NUP53

Figure 4-2. Kap121p-mediated import is specifically inhibited by the overexpression of NUP53. CNP53 (pCUP-NUP53) and W303 (containing the plasmid pCUP1) cells expressing either the Kap121p substrate, Pho4-GFP, or a Kap95p substrate, cNLS-GFP, were grown in the presence (+Cu^{2+}) or absence (-Cu^{2+}) of 0.5 mM copper sulfate for 4 h. The localization of the GFP reporter proteins was examined by direct fluorescence microscopy. Bar=5 μM.

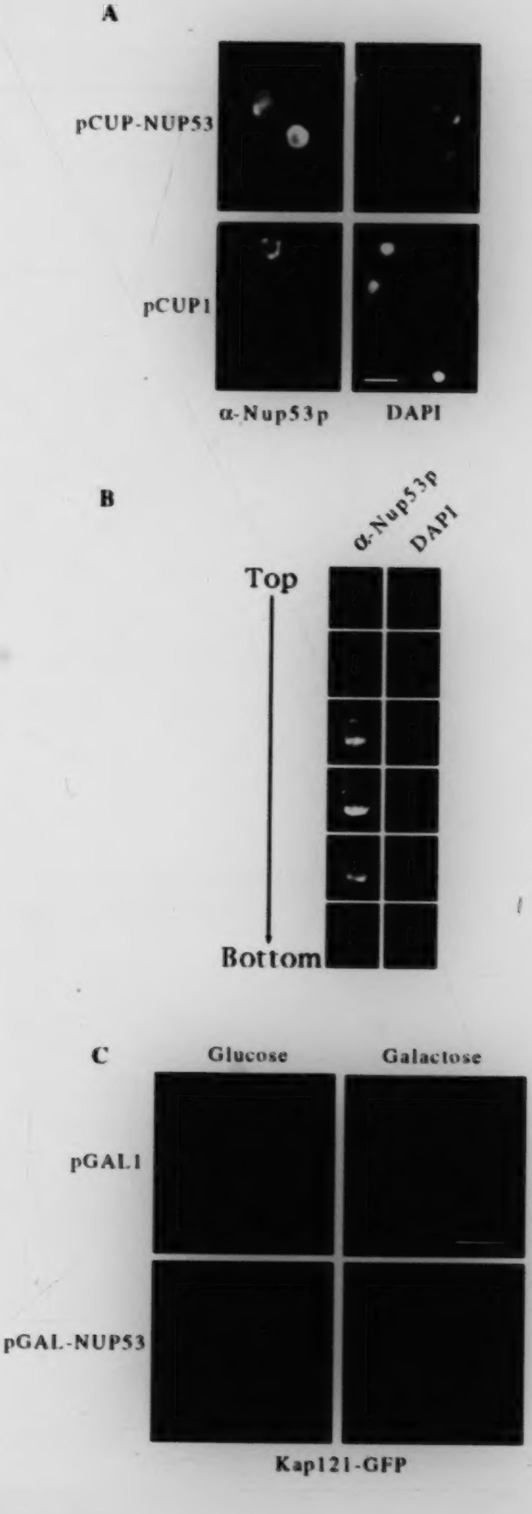
Unlike endogenous levels of Nup53p which display a characteristic punctate perinuclear pattern characteristic of this and other nucleoporins (pCUP1; *Figure 4-3A*), CNP53 cells, induced for 8 h, displayed a continuous perinuclear staining pattern more characteristic of NE staining (Sinissioglou et al., 1996). A similar effect was seen upon galactose-induced overexpression of *NUP53* in cells carrying the pGAL-NUP53 plasmid. Approximately 75% of the cells containing pCUP-NUP53 displayed this pattern. In addition, cells with the strongest staining (~ 20% of the population) often-contained regions of the NE that appeared brighter, and in some cells, Nup53p localized to structures adjacent to the NE. Serial confocal images from such a cell illustrate this staining pattern (*Figure 4-3B*).

Since Nup53p acts as a docking site for Kap121p (see Chapter 3) we examined the effects of *NUP53* overexpression on the localization of Kap121-GFP. Consistent with previous observations, Kap121-GFP is present throughout the cell, but is concentrated at the nuclear periphery in wild type and uninduced cells (*Figure 4-3C*; Seedorf and Silver, 1997). In GNP53 cells expressing Kap121-GFP Nup53p overproduction was induced in galactose containing media. A slight increase in the concentration of Kap121-GFP at the nuclear rim was observed in a few cells of induced cultures, but the overall localization of Kap121p was not dramatically affected.

4.4 Overproduction of Nup53p Induces the Formation of Intranuclear Membranes

We examined the effects of excess Nup53p accumulation on the ultrastructure of the NE by electron microscopy. GNP53 strains were grown for various times in galactose containing media and fixed in potassium permanganate for thin-section

Figure 4-3. Subcellular distribution of overproduced Nup53p. **Panel A**: Wild type W303 cells containing pCUP-NUP53 (CNP53) or pCUP1 were induced in the presence of 0.5 mM copper sulfate for 8 h and processed for immunofluorescence microscopy. Samples were probed with affinity-purified antibodies directed against Nup53p and binding detected with Cy3-conjugated anti-rabbit antibodies. A continuous perinuclear staining pattern was observed in cells overexpressing *NUP53* (pCUP-NUP53) that is distinct from the characteristic punctate perinuclear staining of cells expressing endogenous levels of Nup53p (pCUP1). Note that the exposure time of control cells (pCUP1) was five times that of CNP53 cells. **DAPI** staining was used to determine the location of the nuclear DNA. Bar=5 µm. **Panel B**: Samples of induced CNP53 cells, prepared as described for panel A, were examined by confocal microscopy. Shown are 0.25 µm thick serial-sections through the nucleus showing the location of excess Nup53p and nuclear DNA. **Panel C**: W303 cells containing either pGAL-NUP53, or pGAL1 plasmid and producing the Kap121-GFP reporter were grown in glucose or galactose containing media for 16 h. The subcellular distribution of Kap121-GFP was examined by fluorescence microscopy. Bar=5 µm.


A
pCUP-NUP53
pCUP1
α-Nup53p
DAPI
B
α-Nup53p
DAPI
Top
Bottom
C
Glucose
Galactose
pGAL1
pGAL-NUP53
Kap121-GFP

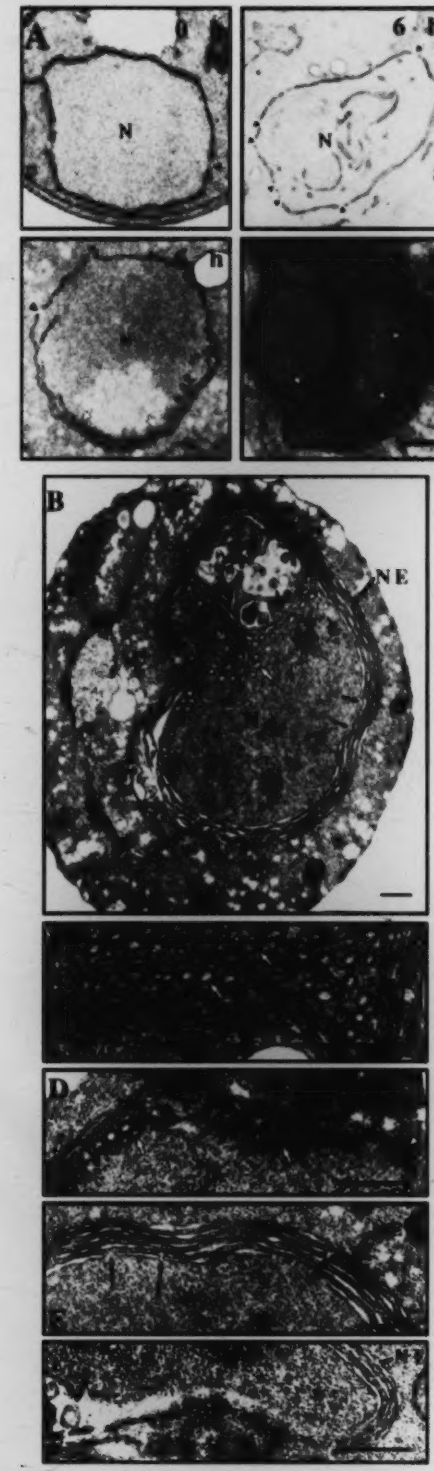
electron microscopy. While this methodology stained the NE membranes intensely, the proteinaceous structures of the NPC are not visible and nuclear pores appear as discontinuities in the NE (*Figure 4-4A;* arrowheads). Prior to induction, cells showed no morphological abnormalities and were indistinguishable from wild type (*Figure 4-4A;* 0 h). However, by 6 h after induction, the nuclei of 30-50% of the cells displayed an extensive intranuclear network of membranes. These membranes appeared initially as tubular or circular shaped membrane-bound cisternae of approximately 50-100 nm in diameter. Starting at 6 h after induction, multiple stacks of double-membrane lamellae appeared to accumulate (*Figure 4-4A;* 6 h). With longer induction times (up to 16 h), the number of the intranuclear lamellar membranes increased, with some cells containing up to 10 layers of double membrane lamellae. In these cells, the multiple lamellar membranes appeared to accumulate in ordered arrays of stacked membranes, occasionally containing pore sized gaps, and occupying large areas of the nucleoplasm (*Figure 4-4A*; 16 h). In addition, the numbers of vesicle-like structures that appear innermost of the lamellar membranes increased. Furthermore, the gradual accumulation of lamellar membranes observed in this time course mirrors the levels of Nup53p (*Figure 4-1B*). In all sections examined, and in serial sections, these membranes appeared to be localized inside the nucleus and surrounded by the NE membrane. Fixing and staining of similar samples with osmium tetroxide and uranyl acetate confirmed the identity of the outer double membrane as the NE. Using this method proteinaceous structures such as the NPCs (arrowheads) and spindle pole bodies (open arrowheads) were present only on the outermost membranes (*Figure 4-4B, 4-4D, 4-4E*), but not within the intranuclear double membrane stacks. These results indicated that the Nup53p-induced membranes were contained within the nucleus.

Since vesicle traffic across the intact NE has never been described, nor have we observed this phenomenon upon overexpression of *NUP53*, the most likely scenario is that the Nup53p induced membranes arise from the inner nuclear membranes. Consistent with this idea, we observed in some rare tangential sections that tubulo-vesicular structures appear to arise from the vicinity of the inner nuclear membrane (*Figure 4-4D*). However, we were unable to see direct continuity between these tubulo-vesicular membranes and the inner nuclear membrane since the morphology was generally not well preserved in these areas. These tubulo-vesicular membranes are likely the precursors of the double membrane lamellae, which are more abundant at later time points. Interestingly, the tubulo-vesicular membranes are occasionally seen aligned in rows in cross section (*Figure 4-4C*; open arrows), and may fuse to form of the lamellar stacks.

4.5 Nup53p Associates with the Intranuclear Lamellar Membranes

The localization of overexpressed Nup53p, observed by immunofluorescence microscopy, at or near the NE, suggested that it was directly associated with the induced intranuclear membranes. This was directly tested by immunoelectron microscopy. GNP53 cells or cells containing an empty plasmid were induced in galactose-containing media for 16 h, fixed and embedded in Epon resin. Sections were labeled with affinity-purified anti-Nup53p antibodies and secondary antibodies conjugated to 10 nm gold particles. Examination of thin-section electron micrographs revealed a high concentration of gold particles along the nuclear periphery in cells overproducing

Figure 4-4. Overexpression of Nup53p induces the formation of intranuclear lamellar membranes. **Panel A**: GNP53 cells were induced in galactose containing media, fixed in potassium permanganate, stained in uranyl acetate, and processed for electron microscopy. This procedure highlights membrane structures but not proteinaceous structures like the NPC. Shown here, are the nuclei of selected cells. Discontinuities in the NE, which likely reflect the position of the NPCs, are indicated by arrowheads. In addition to the single double membrane NE seen prior to induction (**0** h), induced cells contained tubular and circular shaped membranes as well as flattened double membrane lamellae (white arrows) that increase in number upon longer induction (6 h, and 16 h). **N**, nucleoplasm. **Panel B-F**: GNP53 were grown in galactose-containing media for 16 h, fixed and stained in osmium tetroxide and uranyl acetate. This procedure more clearly stains NPCs (arrowheads), and spindle pole bodies (panel D; white arrowhead). The position of the nucleus (**N**), cytosol (**C**), and the NE containing NPCs are indicated. Intranuclear tubulo-vesicular membranes (white arrows) are seen in cross section (panel B, C, D) and in sections parallel to their central axis (panel D). These structures can be seen radiating from the vicinity of the inner nuclear membrane (panel D). Double membrane lamellae having a defined cisternae are visible within the nucleoplasm in stacks often adjacent to the NE. Pores lacking the darkly staining NPCs (arrows) can be seen in cross section (panel B, and E), and *en face* (panel B, and F) through the lamellar membranes. Bar=1 μm.


A
0
6 h
N
N
h
B
NE
D
NE

Nup53p (*Figure 4-5*). The vast majority of the gold particles were seen on the nuclear side of the NE and closely associated with intranuclear structures reminiscent of the membrane stacks and tubulo-vesicular structures discussed above. In contrast, cells containing the empty plasmid did not display this labeling pattern. These observations suggest a physical association between Nup53p and the intranuclear lamellar membranes it induces.

4.6 Pom152p and Ndc1p are Recruited to the Lamellar Membranes.

We also investigated whether other nucleoporins were associated with the intranuclear lamellar membranes. These studies were provoked by the association of Nup53p to these membranes, and the observation that the intranuclear double membrane lamellae appear to contain transcisternal pores reminiscent of those seen in the NE. As seen in *Figure 4-4B*, the intranuclear membranes surrounded a clearly defined cisternae. However, infrequent interruptions of the double lamellar membranes were observed in cross-section within the intranuclear membranes (*Figure 4-4B* and *4E*; arrows). At these sites, a pair of parallel membranes appeared to be linked by a connecting membrane similar in shape to the pore membrane of the NPC. The diameter of the gap (the distance between adjacent membranes) varied between 50-100 nm. In rare sections, these pores contain nucleoplasm, and could be observed in *en face* sections cutting through the lumen of the lamellar membrane (*Figure 4-4F*; arrow). These channels, while similar in diameter to the pores of the NE, did not contain intensely staining NPC structures observed in the NE.

The apparent absence of NPCs in these pores raised the possibility that they represent stalled intermediates in NPC assembly and might contain a subset of nucleoporins. We thus examined the distribution of several nuclear pore proteins by immunofluorescence microscopy in cells overproducing Nup53p. CNP53 cells and W303 cells containing an empty plasmid were induced with copper for 16 h and each was probed with the monoclonal antibodies mAb118c3, which binds the transmembrane protein Pom152p, and mAb414, which binds with FXFG repeat containing nucleoporins (Nsp1, Nup63p, Nup54p, Nup49p). Similarly, cells expressing protein A chimeras of Nup170-pA, Nup59-pA, Nup157-pA, Nic96p-pA, Nup2-pA, Gle1-pA, Ndc1-pA, or Pom34-pA and containing the pCUP-NUP53 or an empty plasmid were induced with copper sulfate and the distribution of the protein A-chimera examined by immunofluorescence. We observed that the punctate perinuclear staining pattern observed in the parent strains was not affected by the overexpression of Nup53p for the majority of these NPC proteins. For example, the punctate perinuclear distribution of Nup170-pA and the mAb414 reactive proteins was observed by conventional fluorescence microscopy and confocal microscopy in both controls and Nup53p overproducing cells.

In contrast, the distribution of two pore membrane proteins, Pom152p and Ndc1-pA, in addition to their perinuclear punctate distribution, displayed a distinct intranuclear staining pattern in 10-20% of the population of cells overexpressing Nup53p (*Figure 4-6*). This percentage concurs with the proportion of cells displaying multiple lamellar stacks. When viewed with confocal microscopy, the additional signal appeared bound by the NE and generally adjacent to the DAPI staining, consistent with their localization to

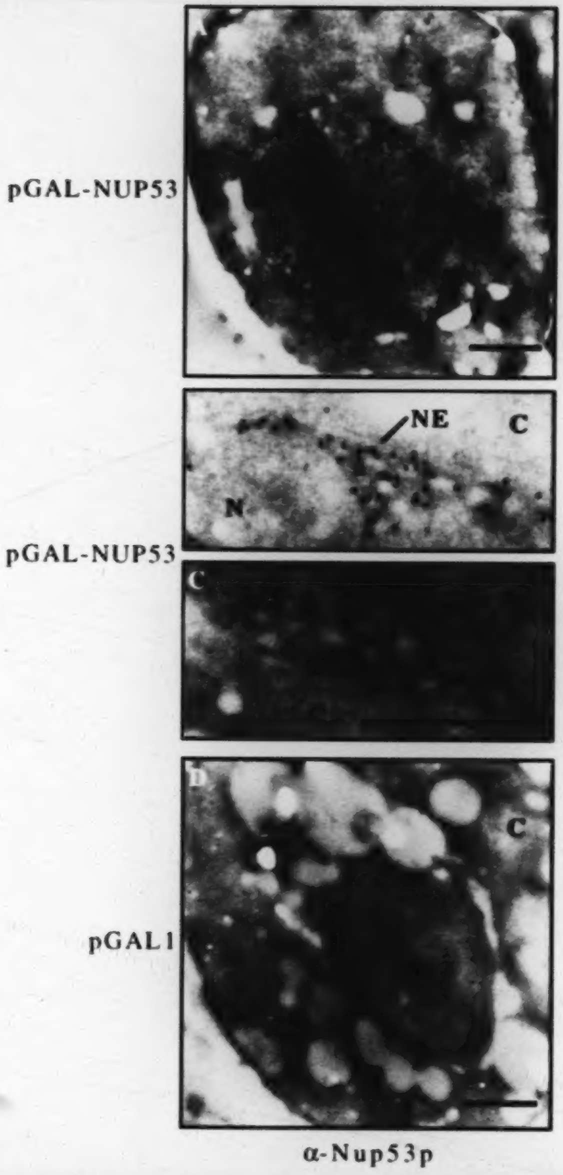
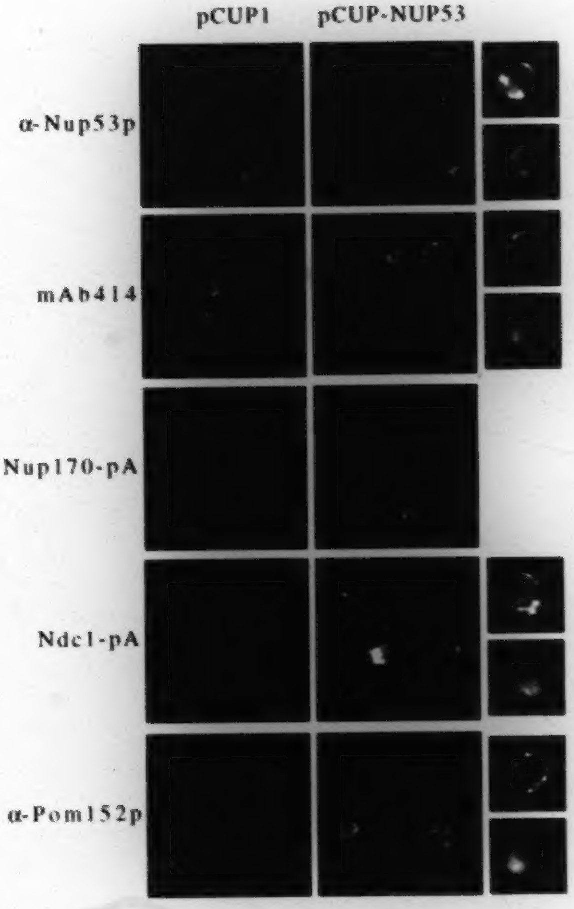


Figure 4-5. Excess Nup53p localizes to the intranuclear lamellar membranes. The localization of Nup53p was examined by immunoelectron microscopy of whole cells. W303 cells containing pGAL-NUP53 (panels A-C), or pGAL1 (panel D) were induced in galactose containing media for 16 h, fixed, embedded and sectioned. Sections were probed with affinity-purified anti-Nup53p antibodies (α-NUP53p) and binding visualized with 10 nm gold-conjugated secondary antibodies. Magnifications of the image shown in panel A are shown in panels B, and C. Nup53p was detected at the nuclear periphery and associated with lamellar membranes and tubulo-vesicular structures. The position of the nucleus (N), and cytoplasm (C) are indicated. NE, nuclear envelope. Bar=1μm.

Figure 4-6. Pom152 and Ndc1p are present in the Nup53p-induced intranuclear membranes. The effect of Nup53p overproduction on the distribution of various NPC proteins was examined by immunofluorescence microscopy. Yeast strains containing the pCUP1, or the pCUP-NUP53 plasmid were grown in media containing 0.5 mM copper sulfate for 16 h and fixed, and permeabilized. DF5 cells were probed with either affinity purified anti-Nup53p (**α-Nup53p**) antibodies or the monoclonal **mAb414** (which binds to FXFG repeat-containing nucleoporins), or mAb118C3 (**α-Pom152**). Binding was detected with Cy3-conjugated secondary antibodies. Strains expressing Nup170-pA (NP170PA), or Ndc1-pA (ProA5-4d) were probed with rabbit IgG followed by Cy3-conjugated donkey anti-rabbit antibodies. Field images were captured by standard fluorescence microscopy. Corresponding single cell confocal microscopy images showing the Cy3 signal (above) and DAPI staining (below) are shown to the right. These images were derived from an optical section through the middle of the nucleus.


pCUP1
pCUP-NUP53
α-Nup53p
mAb414
Nup170-pA
Ndc1-pA
α-Pom152p

the intranuclear lamellar membranes. The intranuclear punctate staining pattern observed for Pom152p indicated that this protein was not uniformly distributed throughout the intranuclear membranes, but suggested that it may be associating with the pores present in these lamellar membranes. In addition, Pom152p reactivity to the monoclonal antibody was more resistant to fixation than in control strains suggesting that Pom152p was more accessible to the antibodies and probably not as tightly associated with other proteins.

4.7 Nup53p Import and Membrane Proliferation are Dependent on Kap121p and Nup170p

The accumulation of Nup53p and its subsequent localization to the inner leaflet of the NE suggested that this protein was actively, and efficiently targeted to the NE. In addition, we hypothesized that Nup53p, either at the NPC or during its import, associates with the inner nuclear membrane and stimulates membrane proliferation. To test these ideas, we examined the role of various karyopherins and nucleoporins on the localization of Nup53p. Previous observations that Kap121p interacts specifically with Nup53p raised the distinct possibility that Nup53p may act as cargo, as well as being a specific docking site for Kap121p at the NPC. To test this idea, a yeast strain containing an allele of *KAP121*, named ***kap121-41***, was isolated that was temperature sensitive for growth. In this strain, the nuclear import of Pho4-GFP was inhibited at both permissive (23°C) (*Figure 4-7A*) and non-permissive (37°C) temperatures. The specificity of the import defect was examined by monitoring the localization of a Kap95p substrate in this mutant.

The cNLS reporter localized to the nucleus at both permissive and non-permissive temperatures (*Figure 4-7A*).

We then tested the effects of this mutation on the localization of overproduced Nup53p by introducing a pCUP-NUP53 plasmid into *kap121-41* and inducing *NUP53* overexpression with copper ions. When examined by immunofluorescence microscopy, this strain displayed a marked accumulation of Nup53p in the cytoplasm at both permissive (*Figure 4-7B*) and non-permissive temperatures. Moreover, thin section electron microscopy analysis revealed that *kap121-41* failed to generate the intranuclear membranes as a result of Nup53p overproduction. In contrast, the localization of overproduced Nup53p and the proliferation of intranuclear membranes were not affected in a strain lacking *KAP123* (*kap123Δ*, *Figure 4-7B*), a karyopherin known to functionally overlap with Kap121p (Rout et al., 1998; Schlendstedt et al., 1998).

Several nucleoporins have been shown to genetically and physically interact with Nup53p (see Chapter 3; Uetz et al., 2000; Tcheperegine, S. and R.W. Wozniak, unpublished data). To determine whether the ability of Nup53p to induce membrane proliferation was dependent on these interactions, we tested whether the Nup53p-interacting nucleoporins played an essential role in the membrane proliferation. For these experiments, we introduced a pCUP-NUP53 plasmid into yeast strains containing a null mutation of *NUP188*, *NUP157*, *NUP170*, *POM152*, or *NUP59* and induced them with copper sulfate. Immunofluorescence microscopy revealed that the excess Nup53p localized to the NE in strains lacking Pom152p (*Figure 4-8*), Nup59p, Nup157p, and Nup188p. In addition, these strains also displayed the characteristic proliferation of membranes when analyzed by thin section electron microscopy. However, in the strain

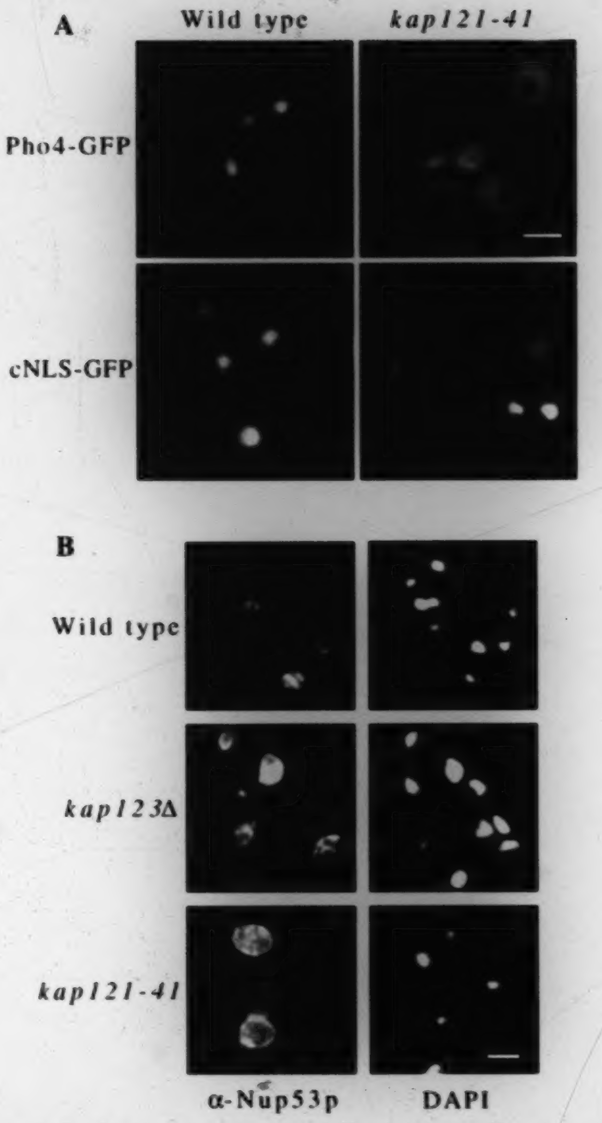


Figure 4-7. The nuclear localization of Nup53p is dependent on Kap121p. **Panel A**: W303 cells (Wild type) and the strain KP121-41 containing as temperature sensitive allele of *KAP121* were transformed with plasmids expressing the Pho4-GFP or cNLS-GFP reporter genes. Cells were grown at 23°C and in each case the subcellular distribution of the reporter proteins was analyzed by fluorescence microscopy. **Panel B**: subcellular distribution of overproduced Nup53p was examined in KP121-41 (*kap121-41*) and 123Δ-14-1 (*kap123Δ*) strains. These transformants and CNP53 cells (Wild type) were induced with 0.5 mM copper sulfate for 16 h, fixed, permeabilized and probed with affinity purified anti-Nup53p antibodies (**α-Nup53p**). Binding was detected with Cy3-conjugated secondary antibodies. The position of the nuclear DNA is visualized by **DAPI** staining. Bar=5 μm.

lacking Nup170p, like in the *kap121-41* strain, Nup53p was seen throughout the cytoplasm and did not accumulate at the NE (*nup170Δ*; *Figure 4-8*). This strain also failed to produce the characteristic lamellar membranes when analyzed by electron microscopy.

4.8 A C-Terminal Amphipathic Helix in Nup53p is Required for Membrane Proliferation

The results described above show that the targeting of overproduced Nup53p is dependent on Kap121p and Nup53p's interaction with Nup170p. Since the subcellular localization of Kap121p and Nup170p is not altered in Nup53p overproducing stains it is unlikely that they accompany Nup53p into the lamellar membranes, but rather that they may play a role in the membrane association of Nup53p at the NPC. To understand the mechanism by which Nup53p associates with the membranes we have investigated the biochemical basis for Nup53p's association with the intranuclear membranes and defined a region in Nup53p that is required for membrane proliferation.

To examine the biochemical basis of Nup53p's association with the nuclei, NEs were isolated from CNP53 cells and W303 cells. NEs were extracted with 0.1 M sodium carbonate, pH 11.4 to separate peripheral membrane components in the soluble pool from those tightly associated with the membrane pellet, such as the integral membrane proteins. As shown in *Figure 4-9A*, Nup53p was partially extracted from the wild type NEs. However, a significant proportion of Nup53p remained associated with the membrane fractions along with the integral membrane protein Pom152p (*Figure 4-9A*). Likewise, extraction of NEs derived form CNP53 cells revealed that approximately 50%

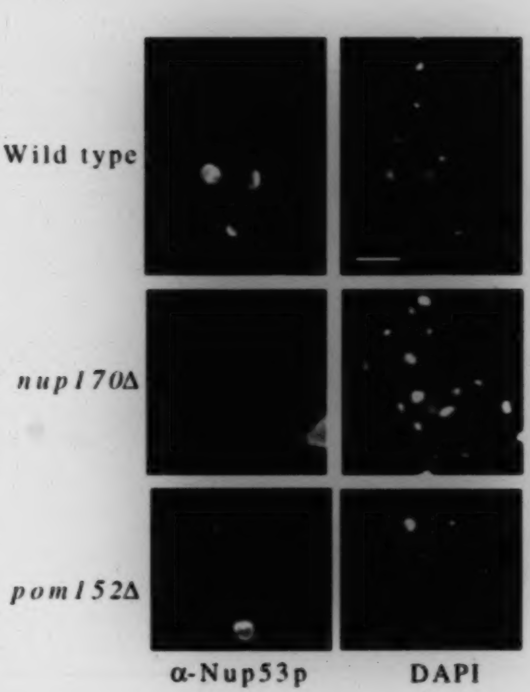


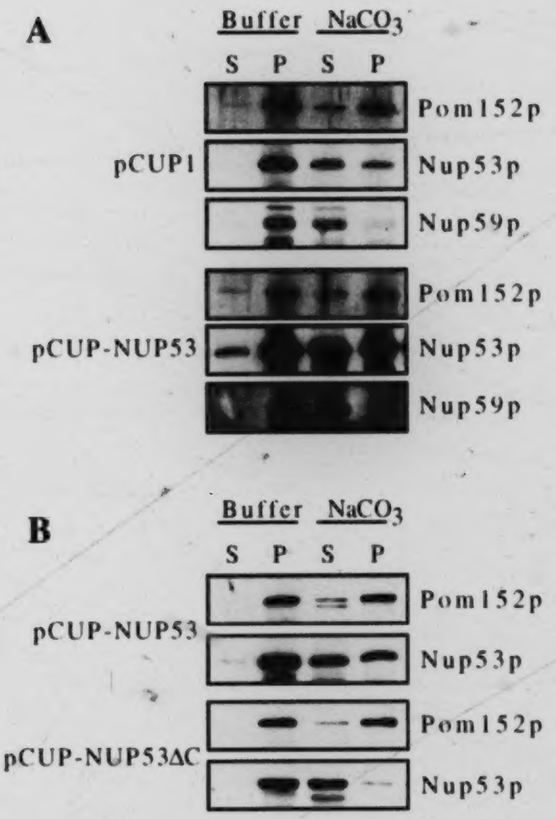
Figure 4-8. Overproduced Nup53p is mislocalized in nup170Δ cells. The subcellular distribution of overproduced Nup53p was examined in strains lacking Nup170p and Pom152p. NP170-11.1 (*nup170Δ*) and PMY17 (*pom152Δ*) strains were transformed with the pCUP-NUP53 plasmid. These strains and CNP53 (Wild type) were induced in 0.5 mM copper sulfate for 16 h, and processed for immunofluorescence microscopy. The localization of overproduced Nup53p was determined using affinity purified anti-Nup53p antibodies (α-Nup53p). Binding was detected with a Cy3-conjugated secondary. DAPI indicates the position of nuclear DNA. Bar=5 μm.

of the excess Nup53 was resistant to sodium carbonate extraction (*Figure 4-9A*) and remained associated with the membranes. Other peripherally associated nucleoporins, like Nup59p (*Figure 4-9A*) were quantitatively extracted from the NEs.

The resistance of endogenous, and to a greater degree overproduced Nup53p to carbonate extractions suggested that it is more tightly associated with the membrane than other nucleoporins with which it interacts, including Nup59p. However, it is unlikely that Nup53p transverses the membrane as it lacks a predicted transmembrane segment and it does not show the same level of resistance to extraction that is observed with integral membrane proteins including Pom152p (Wozniak et al., 1994; *Figure 4-9A*, and *9B*). A strong association with the periphery of the membrane could explain Nup53p's resistance to sodium carbonate extractions either through its interaction with a transmembrane protein or directly with the nucleoplasmic leaflet of the bilayer. Analysis of the secondary structure of Nup53p revealed that its C-terminal 26 amino-acid residues could form an amphipathic α-helix capable of mediating its interaction with a membrane (*Figure 4-10*). To address this question, a pCUP-NUP53ΔC plasmid was introduced into a *nup53*Δ null strain and induced with copper ions. The induction of these cells (CNP53ΔC) resulted in the overexpression of Nup53p lacking the C-terminal 26 amino acids (Nup53ΔCp) at levels comparable to those of full length Nup53p in induced CNP53 cells. Nuclei were isolated from induced CNP53ΔC and CNP53 cells. Sodium carbonate extractions of these nuclei showed that unlike full-length Nup53p, Nup53ΔCp was quantitatively extracted from the membrane fractions (*Figure 4-9B*). Pom152p was not extracted from the membrane fraction of either source of nuclei. Furthermore, thin section EM analysis of cells overproducing Nup53ΔCp did not stimulate the formation of lamellar membranes (P. Lusk and R. Wozniak, personal communication). While failing to tightly bind membranes and stimulate membrane proliferation, Nup53ΔCp does associate with NPCs and is imported into the nucleus, however it does not appear to bind the inner nuclear membrane(P. Lusk and R. Wozniak, personal communication). These results indicate that the putative C-terminal 26 amino-acid residue α-helix is necessary for the formation of intranuclear lamellar membranes.

4.9 Endogenous Nup53p is Mislocalized in *kap121* and *nup170Δ* Mutant cells

The observation that Kap121p and Nup170p were necessary for the proper localization of overproduced Nup53p raised the interesting possibility that these components may also be required for the proper localization of endogenously expressed Nup53p. Thus, the distribution of Nup53p in *kap121-41*, *nup170Δ*, and *pom152Δ* mutant cells was compared to that in wild type cells by indirect immunofluorescence microscopy (*Figure 4-11*). While Nup53p appeared to be entirely localized at the nuclear periphery in wild type and *pom152Δ* cells, a discrete cytoplasmic accumulation was observed in both *kap121-41* and *nup170Δ* mutant cells. These results indicate that the localization of endogenous Nup53p at the NE is, in part, dependent on the function of Kap121p and Nup170p.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S
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pCUP1
Pom152p
Nup53p
Nup59p
pCUP-NUP53
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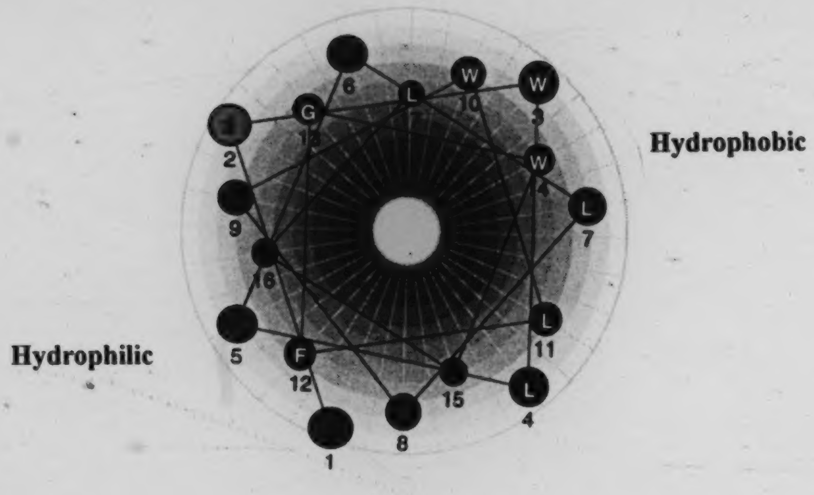
Nup59p
B
Buffer
NaCO3
S
P
S
P
pCUP-NUP53
Pom152p
Nup53p
pCUP-NUP53ΔC
Pom152p
Nup53p

Figure 4-9. Biochemical analysis of Nup53p. **Panel A**: NEs were isolated from CNP53 cells (pCUP-NUP53) and W303 cells containing pCUP1 following 4 h of growth in media containing 0.5 mM copper sulfate. Suspended NEs were treated with buffer alone, or extracted with sodium carbonate (NaCO$_3$), pH 11.4. Samples were then centrifugated to produce a supernatant fraction (S) and a membrane-containing pellet (P). Samples were analyzed by Western blotting using rabbit polyclonal antibodies directed against Nup53p and Nup59p, and the monoclonal antibody mAB118C3 (α-Pom152p). Binding was detected with HRP-conjugated secondary antibodies and ECL. **Panel B**: The same analysis was performed on nuclei isolated form induced CNP53 (pCUP-NUP53) cells or the CNP53ΔC (pCUP-NUP53ΔC) strain expressing a truncation of Nup53p lacking its last 25 amino-acid residues.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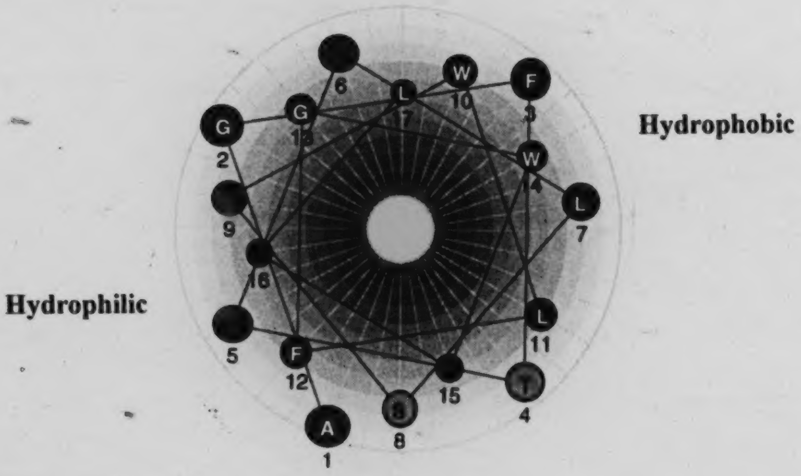
Nup53p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Nup59p

Figure 4-10. Nup53p and Nup59p are predicted to have an amphipathic α-helix at their C-terminus. Helical wheel representation of the C-termini of **Nup53p** (amino-acid residues 458-475) and **Nup59p** (amino-acid residues 511-528). Hydrophobic and hydrophilic faces of both α-helices are indicated.

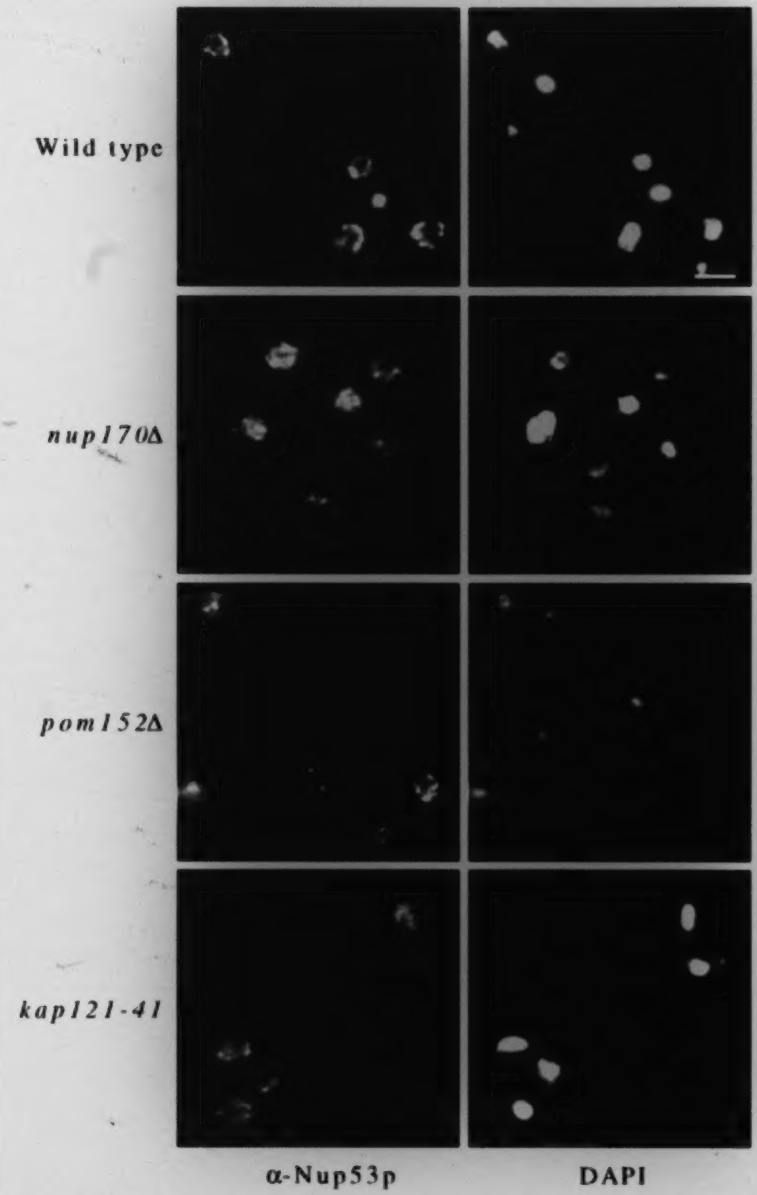

Figure 4-11. Nup170p and Kap121p are required for the efficient localization of endogenous Nup53p at the NE. The subcellular distribution of endogenously expressed *NUP53* was examined in W303 (Wild type), NP170-11.1 (*nup170Δ*), PMY17 (*pom152Δ*), and KP121-41 (*kap121-41*) strains. Exponentially growing strains were fixed, permiabilized, and probed with affinity-purified anti-Nup53p antibodies (**α-Nup53p**). Binding was detected with Cy3-conjugated anti-rabbit antibodies. The position of nuclear DNA was determined by **DAPI** staining. Bar=5 μm.

4-10 Discussion

Nup53p is unique amongst nucleoporins that bind karyopherins in that it appears to provide a specific docking site for Kap121p (see Chapter 3; Damelin and Silver, 2000). Mutations in *NUP53* have been shown to affect the subcellular distribution of Kap121p as well as Kap121p-mediated import (see Chapter 3). Consistent with these observations, we have shown that increasing cellular levels of Nup53p inhibited the import of the Kap121p substrate Pho4p, without affecting the localization of a Kap95p import substrate. These results support our hypothesis that at least some karyopherins follow different, and potentially regulated pathways through the NPC.

The mechanism by which excess Nup53p affects Kap121p-mediated import remains undefined. Our observations that increased levels of Nup53p induced the formation of lamellar membranes suggested that these could provide a physical barrier to import. However, the effective import of a cNLS-GFP reporter indicated that the inhibition of Pho4p import was not due to physical constraints, which could affect all transport pathways. The specific interaction of Nup53p with Kap121p also suggested that Nup53p might be competing with Pho4p-GFP reporter for Kap121p binding. Our observations that Nup53p was efficiently targeted to the interior of the nucleus in a Kap121p-dependent manner (*Figure 4-7, and 4-11*), suggested that the overexpression of *NUP53* creates a pool of Kap121p "cargo" that may compete with other Kap121p substrates for binding to the karyopherin, and consequently reducing the efficiency of their import. A nucleoporin-stimulated release of cargo from a transport factor has been previously observed (Rexach and Blobel, 1995; Bastos et al., 1996). However, the efficient localization of Nup53p to the nucleus did not appear to saturate the Kap121p import system. Therefore, a more likely scenario might be that Nup53p interferes with the dynamics of Kap121p-mediated import at the NPC. Consistent with this idea, we observed that overexpressed Pho4-GFP localized to the nuclear periphery in a number of cells (*Figure 4-2*) and suggests that a step in the translocation of the Kap121p/Pho4-GFP complex through the NPC is rate limiting in the presence of excess Nup53p.

Overproduced Nup53p accumulates within the nucleus and is visible by immunoelectron microscopy in association with the lamellar membranes and the tubulo-vesicular membrane structures inside the nucleus. At no time did we observe a diffuse nuclear pool of Nup53p, suggesting that it associates with the membrane either at the pore domain or the inner nuclear membrane. The import of excess Nup53p, and its ability to induce membrane proliferation is dependent on the function of Kap121p and Nup170p, but not other NPC proteins including two integral proteins of the pore membrane Pom152p and Pom34p, and Nup157p, a nucleoporin highly similar to Nup170p. In both *Kap121-41* and *nup170*Δ mutant cells, Nup53p was localized to the cytoplasm and failed to induce lamellar membrane formation. This result demonstrates that the production of membranes requires efficient targeting of overproduced Nup53p and that this targeting is mediated by Kap121p. The role of Nup170p, however, in not as clear. Nup170p may be involved in the insertion, maintenance, or targeting of Nup53p to the membranes, or in the actual import process. The import of Pho4-GFP at near wild type rates in a strain lacking *NUP170* suggests that the mislocalization of Nup53p in this strain is not linked to a defect in Kap121p import (Shulga et al., 2000; Lusk, P., Marelli, M., and R.W. Wozniak, unpublished observations). Since Nup170p acts as a binding site for Nup53p at the NPC, Nup170p may play a direct role in mediating the association of Nup53p with the intranuclear membranes.

The nuclear import of Nup53p and its association with the inner nuclear membrane leads to the proliferation of membrane structures within the nucleus. The membrane expansion appears to originate at regions adjacent to the NE where tubulo-vesicular membranes were visible at the earliest stages of membrane proliferation (*Figure 4-4A, 4-4D*). These tubulo-vesicular structures may fuse together to form extended sheets of flattened, membrane-enclosed cisternae, which are structurally similar to the overlying NE. The double membranes of the lamellae were occasionally attached by a connecting membrane forming transcisternal pores. But unlike in the NE, these pores did not appear to contain NPCs, nor could we detect the presence of the vast majority of nucleoporins in these membranes by immunofluorescence. Notable exceptions were the integral pore-membrane proteins Pom152p and Ndc1p (*Figure 4-6*). In a subset of cells overproducing Nup53p, Pom152p and Ndc1p localized to intranuclear structures suggesting that they may associate with the transcisternal pores. While we were unable to show a direct association between Pom152p, Ndc1p and the pores of the intranuclear lamellar membranes, the selective recruitment of these components but not of other NPC markers to localized *foci*, suggests that these represent stalled intermediates in NPC assembly. This incomplete assembly is likely due to a lack of availability of critical nucleoporins rather than topological constraints as intranuclear NPCs have been previously observed (Wente and Blobel, 1993; Aitchison et al., 1995). For Pom152p (Strambio-de-Castillia et al., 1995) and possibly Ndc1p, excess amounts of these proteins located at the endoplasmic reticulum may provide a source of material for their recruitment to the forming membranes.

Interestingly, both Ndc1p and Pom152p have been shown to interact with Nup53p by two-hybrid analysis (Uetz et al., 2000). This may explain the recruitment of these components to the lamellar membranes. However, it is unlikely that Ndc1p and Pom152p anchor Nup53p to the induced intranuclear membranes since the endogenous levels of these proteins appears to be unaltered in cells overexpressing Nup53p. Consistent with this idea, the formation of intranuclear lamellae upon *NUP53* overexpression was observed in strains lacking *POM152*. Thus, the association of excess Nup53p with the membranes may occur through an as of yet unidentified protein, or by a direct interaction with the membrane. In either case, Nup53p's association with the membranes and its ability to induce membrane proliferation was found to be dependent on its last 26 amino-acid residues (*Figure 4-9*). Consequently, a mutant lacking this region, Nup53ΔCp, localized to the NPCs and excess accumulated into the nucleus (Lusk P., Marelli, M., and R.W. Wozniak, unpublished data). These results suggested that this region is not necessary for the targeting of Nup53p to the nucleus.

Extractions of NEs derived from cells overexpressing *NUP53* indicated that Nup53p was resistant to alkaline extractions suggesting a tight association with the nuclear membranes than Nup59p, or Nup170p (*Figure 4-9*). The interaction of Nup53p with the membrane fraction was found to be mediated by a C-terminal sequence of amino-acid residues predicted to form an amphipathic α-helix. This region of Nup53p may interact directly with the endoplasmic leaflet of the pore-membrane or the inner nuclear membranes. Its association is specific for these membranes. Overproduced

Nup53p that is mislocalized in *kap121-41*, and *nup170Δ* mutants does not appear to associate with other intracellular membranes, but rather is seen diffusely distributed throughout the cytoplasm. An exception to this is seen in a subpopulation of cells containing the highest concentrations of Nup53p and where we observed that Nup53p accumulates in the cytoplasm and appears to associate with the plasma membrane. This observation supports the idea that Nup53p interacts directly with the membranes since the plasma membrane is unlikely to contain receptors for Nup53p. A notable paradigm for the specific and regulatable interaction of an amphipathic α-helix with a defined membrane system exists in the endoplasmic reticulum-Golgi network. Here, the association of ADP-ribosylation factors (ARFs) with the membrane is mediated by an N-terminal, amphipathic α-helix whose insertion into the membranes is mediated by the ARF's binding to GTP, which is stimulated by a guanoidine nucleotide exchange factor present on the membrane (see Jackson and Casanova, 2000).

The intranuclear membrane structures that are induced by Nup53p overproduction are unique to this yeast nucleoporin. However, a number of other structural abnormalities in the NE membrane are induced by changes in the stoichiometric balance of nucleoporins. For example, cells lacking Nup116p or Gle2p/Nup40p, or in mutants of Nup188p (Nehrbass et al., 1996) or Nup145p (Wente and Blobel, 1994), produce what appear to be local extensions of the outer nuclear membrane that seal over the NPCs and form herniations. Likewise, depletions of Nup170p in a *pom152Δ* null strain led to abnormally shaped nuclei that contain finger-like projections and invaginations (Aitchison et al., 1995a; Nehrbass et al., 1996). These morphological defects were also observed in mutants of *NUP1*, *NUP84*, and *NUP85* (Bogerd et al., 1994; DeHoratius and Silver, 1996; Goldstein et al., 1996; Sinissioglou et al., 1996).

The structures most closely related to those seen in Nup53p overproducing strains are layers of lamellar membranes, termed karmellae, that were first seen upon overexpression of the integral endoplasmic reticulum protein HMG-CoA reductase 1 (Wright et al., 1988). Similar structures have also been observed in yeast upon overexpression of integral membrane proteins like the human lamin B receptor (Smith and Blobel, 1994), and the canine 180-kD ribosome receptor (Becker et al., 1999). These karmellae appear to arise from the outer nuclear membrane, forming multiple layers of double membrane lamellae that surround large portions of the nucleus. However, these structures appear to be distinct from those induced by the overexpression of Nup53p and may be formed by separate mechanisms. Karmellae are topologically distinct being present only in the cytoplasm. In addition, karmellae do not appear to have transcisternal pores, nor tubulo-vesicular structures.

The intranuclear membrane proliferation observed by the overproduction of Nup53p, is not unprecedented. The overexpression of Nup153p in BHK cells also was reported by Bastos et al. (1996) to result in an intranuclear proliferation of membranes. Like Nup53p, Nup153p appears to associate with the inner nuclear membrane and the induced membrane arrays that appear to be composed of tubulo-vesicular membrane structures similar to, but much less extensive than those seen by the overexpression of Nup53p. The N-terminal domain of Nup153p contains an M9-like NLS, capable of functioning autonomously as an NLS (Nakielny et al., 1999) and an N-terminal segment (amino-acid residues 2-144) that is capable of associating with the inner nuclear membrane (Enarson et al., 1998). This region (amino-acid residues 39-57), is also capable of forming an amphipathic α-helix similar to the C-terminus of Nup53p. The similarities between these proteins suggest that several steps of NE biogenesis may be conserved between yeast and higher eukaryotes. Furthermore, these seemingly unrelated nucleoporins might have a common role in the biogenesis of the NE and the assembly of NPCs. In contrast, the overexpression of Nup59p failed to produce the intranuclear membrane lamellae despite the amino-acid sequence similarity at the C-terminus between Nup59p and Nup53p (*Figure 3-1,* and *4-10*). The C-terminus of Nup59p is also predicted to form an amphipathic α-helix, similar to that of Nup53p (*Figure 4-10*). The inability of overproduced Nup59p to promote the intranuclear lamellae may be due, in large part, to its inability to associate with the NPC or be imported into the nucleus.

The results of the Nup53p overexpression studies have provided some insight into the function of Nup53p and the dynamics of its association with the NPC. Nup53p acts as a specific binding site for Kap121p. But in addition to this role it appears that Nup53p is also recognized as cargo by Kap121p. One interpretation of these results is that Kap121p targets newly synthesized Nup53p to its sites of integration within the NPCs, perhaps functioning in the maintenance of existing NPCs or in the formation of new NPCs. As described above, Nup170p plays a critical role in this process as well. Consistent with this idea, we have shown that endogenous Nup53p is mislocalized in strains containing the mutant alleles of *kap121-41* or *nup170Δ*(see *Figure 10*). These results may highlight a role for karyopherins in the biosynthesis of NPCs. Other nucleoporins including Nic96p (Grandi et al., 1995), and Nup153p (Nakielny et al., 1999) have been shown to contain an NLS. These regions may mediate interactions with the karyopherins in transport. It will be interesting to see whether these sequences and their corresponding karyopherins function to target these nucleoporins to the NPC.

The association of Nup53p with the NE and the proliferation of membranes that follow may be indicative of a broader range of physiological functions of Nup53p and possibly other nucleoporins. For example, the Nup53p-induced membrane expansion may reflect a general role for the NPCs in regulating the expansion of the NE, an event that must occur every cell cycle in response to changes in nuclear volume. Nup53p overexpression may provide a tractable system by which to investigate the mechanism that drives membrane proliferation. Moreover, the recruitment of Nup53p in yeast, and Nup153p in vertebrates, to the inner nuclear membranes raises many questions as to their role at this location. One possibility is that they could function to seed NPC formation, possibly at localized foci established by the underlying chromatin. Subsequent changes in the membrane structure, including the recruitment of membrane proteins, could lead to the formation of transcisternal pores and the assembly of NPCs. Such inside out pore formation could occur throughout the cell cycle in yeast (Winey et al., 1997), and during S-phase in higher eukaryotes, when NPC numbers double (Maul et al., 1972). This mechanism could provide a means of linking specific genes to NPCs (Blobel, 1985).

Chapter 5

Perspectives and Future Studies

5.1 Synopsis

We have used genetic, biochemical, and cell biological approaches to identify yeast nucleoporins and examine their role in nucleo-cytoplasmic exchange. One of these, Nup53p, is a member of an NPC subcomplex containing the nucleoporins Nup59p and Nup170p and acts a specific binding site for the karyopherin Kap121p. *NUP53* mutations affect the subcellular distribution of Kap121p, and Kap121p-dependent nuclear import. In addition, Nup53p overexpression inhibits Kap121p-mediated import and induced the formation of intranuclear lamellar membranes suggesting a role for this protein in membrane biogenesis.

5.2 Perspectives on Specific Binding Sites for Karyopherins at the NPC

The NPCs provide the sole site through which nucleocytoplasmic exchange can take place. Considerable attention has been dedicated to the identification of nucleoporins, the determination of their nearest neighbor interactions, and the role that these components play in the transport process. With a complete inventory of both nucleoporins and karyopherins in yeast, the field is poised to reveal the molecular mechanisms of nuclear transport. Although the interaction of karyopherins with several nucleoporins has been previously observed, the identification of preferred binding sites for specific karyopherins suggests that different import pathways through the NPC exist. One example of this is represented by the specific interaction between Nup53p and Kap121p (see Chapter 3). However, some nucleoporins have been found to interact with several different classes of karyopherins, suggesting the presence of a common import pathway (Aitchison et al., 1996; Rout et al., 1997; See Chapter 3). The observation that strains lacking *NUP53* are viable (and Kap121p null strains are lethal), as well as the identification of other nucleoporins that interact with Kap121p (see Chapter 3; Damelin and Silver, 2000), indicates that Kap121p may choose between import pathways when its preferred sites are unavailable. The combination of 'common' and 'specific' transport pathways for karyopherins may serve to reduce the competition between β-karyopherins for binding sites on the NPCs and ultimately increase the efficiency of import.

The molecular mechanics behind the translocation of karyopherins and their cargos remains undefined. But the identification of an NPC subcomplex containing Nup53p, Nup170p, and Nup59p, which mediates the interaction between the NPC and Kap121p, may provide a system by which to elucidate some of these events. It will be interesting to examine the molecular dynamics of Kap121p docking to this subcomplex and its effects on the interactions between its components. The molecular interactions between these components may be monitored both *in vivo*, with the development of fluorescence based imaging technologies (Clegg, 1996; Damelin and Silver, 2000), and *in vitro*, using binding assays. Such experiments may shed some light on whether Nup53p provides a static binding site for Kap121p, or whether the interactions between Nup53p, Nup170p, and Nup59p are modulated. In fact, preliminary evidence suggests that Nup170p can disrupt the interaction between Kap121p and Nup53p (Lusk, P. and R.W. Wozniak unpublished data). Thus, Nup170p binding to Nup53p may change Nup53p's affinity for Kap121p and release it to adjacent binding sites deeper within the pore. This may be repeated several times with other Kap121p-interacting nucleoporins and ultimately terminating with the release of Kap121p into the nucleus. How many proposed binding-release reactions are required to move through the pore remains to be defined. This, however, may not be easy to determine due to transient interactions that may occur.

5.3 On the Control of Import Pathways at the NPC

The existence of specific binding sites at the NPC for a specific class of karyopherins may also create the potential for differential control of their import pathways. The modulation of the interaction between a karyopherin and a specific binding site on the NPC may provide a mechanism by which to attenuate or promote the translocation of a karyopherin and its substrates. As discussed earlier, one possible modification by which a transport pathway may be altered is through the reversible phosphorylation of a nucleoporin (see Chapter 3). In yeast, Nup53p is specifically phosphorylated in mitosis. This event correlates with a significant reduction in the localization of Kap121p at the NPC and suggests that the modification of Nup53p affects its interaction with Kap121p. In addition, recent data suggests that Pho4p, a Kap121p import substrate, is also mislocalized during mitosis (Lusk, P., Marelli, M. and R.W. Wozniak, unpublished observations). Although the mechanism behind this regulation remains to be defined, the mitotic phosphorylation of Nup53p may affect the affinity of Nup53p for Kap121p, either directly, or via a modification of the interactions between itself and Nup170p or Nup59p (or other NPC components). Since the phosphorylated state of Nup53p is transient, it will be important to first define the phosphorylation sites of Nup53p in order to examine the effects of this modification on its nearest neighbor interactions and on Kap121p binding. One must keep in mind, however, that M-phase phosphorylation may occur on a number of nucleoporins and affect a number of import pathways. The identification of other phosphorylated yeast nucleoporins would implicate phosphorylation as a common regulator of nucleocytoplasmic transport at the NPC.

5.4 On Nuclear Envelope Biogenesis

The overexpression of Nup53p resulted in a series of related phenotypes that may reflect functional properties of Nup53p. First amongst these is the dramatic accumulation of intranuclear membranes as a result of increased cellular amounts of Nup53p. While it is unlikely that Nup53p alone may be involved in triggering NE biogenesis, an exciting possibility is that overproduction of Nup53p stimulates the activation of a pathway that regulates the expansion of the NE (reviewed in Nunnari and Walter, 1996). Thus, a relative increase in the amount of membrane-associated Nup53p is countered by a similar increase in the synthesis of membranes in order to accommodate the excess Nup53p. This system may provide an invaluable source for the identification and characterization of factors involved in the synthesis of the NE, and in deciphering the regulatory mechanisms that control these events.

Does endogenous Nup53p play a role in the expansion of the NE? An intriguing possibility is that as a cell prepares for division a subpopulation of Nup53p associates with the inner NE stimulating the biosynthetic pathway and the production of new membranes. This re-localization may occur by continuous synthesis of Nup53p, which slowly accumulates in the NE, or by a regulatory modification that may alter the interactions between Nup53p and other NPC components and results in the internalization of the protein. This event could produce the elevated levels of Nup53p that stimulate a slow proliferation of membranes. Nup53p has been shown to be reversibly phosphorylated during mitosis, and may represent such a regulatory event (see Chapter 3). The increased concentration and association with the inner nuclear envelope may stimulate a localized proliferation of membranes. The subsequent decrease in the concentration of Nup53p on the cytoplasmic aspect of the NE may explain the reduced localization of Kap121p at the nuclear envelopes in late M-phase cells (see Chapter 3).

The proliferation of membranes as a result of overexpression of integral membrane proteins has been previously described (Wright et al., 1988; Vergeres et al., 1993; Smith and Blobel, 1994; Parrish et al., 1995; Becker et al., 1999). However, the association of Nup53p to the NE appears to be mediated by a C-terminal domain predicted to form an amphipathic α-helix. Deletion of this region affected the interaction of Nup53p with the membranes and prevented the formation of the intranuclear membranes suggesting that the membrane proliferation is dependent on its interaction with the NE. Interestingly, similar effects were observed upon overproduction of Nup153p in vertebrate cells (Bastos et al., 1996), indicating that it may also contain a region that directly or indirectly interacts with the membranes. Sequence analysis of other nucleoporins, and overexpression of these may be performed in order to identify other nucleoporins with a role in NE biogenesis.

5.5 On Alternate Functions of Nup53p

The intranuclear membranes of Nup53p-overexpressing cells may also be a unique source of inner NE membranes, that could be used to identify integral membrane proteins and peripherally-associated components. Since a nuclear lamina is absent in yeast, such components may play basic roles in maintaining the stability of the NE and in the structural organization of chromatin; as a consequence these may be involved in vital functions such as chromosome replication and modulation of gene expression. An understanding of the composition of the inner nuclear membrane will go a long way to defining the role that the NE has on the organization of the nucleoplasm, distribution of NPCs, and nucleocytoplasmic transport.

5.6 On Nuclear Pore Complex Assembly

The biogenesis of NPCs is required every cell cycle in order to maintain a constant number of NPCs from generation to generation. However, the mechanism behind the concerted assembly of the NPCs and their insertion into the NE remains undefined. Interestingly, the intranuclear lamellae that develop as a result of Nup53p overproduction appeared as paired membranes with an intermittent lumen resembling the overlying NE, but lacking NPCs. This observation indicated that the formation of the NE and the integration of NPCs are separable and unique events, and possibly require different components and regulatory pathways. The overproduction of NEs, although a first step in making membrane available for new NPCs, was not sufficient to trigger their assembly. The presence of transcisternal pores, concomitant with the recruitment of the integral membrane proteins Pom152p and Ndc1p at localized foci, suggests that these nucleoporins could be acting as "seeds" for pore formation. It has been a long held belief that the integral membrane proteins of the NPC may associate in the NE and serve as a template for NPC formation (Gerace et al., 1992; Hallberg et al., 1993; Wozniak et al., 1994; Macauley and Forbes, 1996). Whether Pom152p and Ndc1p are physically associated with the pores in the lamellar membranes remains to be examined directly.

However, other NPC components including a subclass of FXFG repeat containing nucleoporins, and other structural nucleoporins like Nup157p, Nup170p, Nup59p, Nic96p, and Nup188p did not appear to be recruited into the lamellar membranes. This could be a result of a lack of availability of these components to the forming pores. Overexpression of other structural NPC components may result in the recruitment of these proteins into the lamellar pores and could provide a system to dissect the order of events required to form mature NPCs. Thus far, we have only investigated a small subset of nucleoporins. A more extensive analysis, together with data from the composition of these membranes, may delineate the components that are necessary for the formation of NPCs.

The dramatic accumulation of intranuclear membranes as a result of Nup53p overproduction is dependent on the proper targeting of excess Nup53p to the membranes and its association with the NE. Interestingly, the targeting of Nup53p to the nucleus of both overexpressed and endogenous Nup53p is dependent in large part to the function of Kap121p. This raises the exciting possibility that karyopherins may be involved in the biogenesis of NPCs by delivering nucleoporins to the NPC. Since a full two-thirds of the yeast nucleoporins have been shown to interact with karyopherins it may be interesting to investigate their localization in a variety of karyopherin mutant strains. These studies may determine whether other nucleoporins are delivered by similar mechanisms and begin to define the process of NPC assembly in yeast.

Chapter 6

References

Adam, S.A., R.S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J Cell Biol.* 111:807-16.

Adam, S.A., and L. Gerace. 1991. Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell.* 66:837-47.

Adam, E.J., and S.A. Adam. 1994. Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J Cell Biol.* 125:547-55.

Aitchison, J.D., M.P. Rout, M. Marelli, G. Blobel, and R.W. Wozniak. 1995. Two novel related yeast nucleoporins Nup170p and Nup157p: complementation with the vertebrate homologue Nup155p and functional interactions with the yeast nuclear pore-membrane protein Pom152p. *J Cell Biol.* 131:1133-48.

Aitchison, J.D., G. Blobel, and M.P. Rout. 1995b. Nup120p: a yeast nucleoporin required for NPC distribution and mRNA transport. *J. Cell Biol.* 131:1659-75.

Aitchison, J.D., G. Blobel, and M.P. Rout. 1996. Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science.* 274:624-7.

Akey, C.W., and D.S. Goldfarb. 1989. Protein import through the nuclear pore complex is a multistep process. *J Cell Biol.* 109:971-82.

Akey, C.W., and M. Radermacher. 1993. Architecture of the Xenopus nuclear pore complex revealed by three- dimensional cryo-electron microscopy. *J Cell Biol.* 122:1-19.

Amberg, D.C., A.L. Goldstein, and C.N. Cole. 1992. Isolation and characterization of RAT1: an essential gene of Saccharomyces cerevisiae required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.* 6:1173-89.

Arts, G.J., S. Kuersten, P. Romby, B. Ehresmann, and I.W. Mattaj. 1998. The role of exportin-t in selective nuclear export of mature tRNAs. *Embo J.* 17:7430-7441.

Atkinson, N.S., R.W. Dunst, and A.K. Hopper. 1985. Characterization of an essential Saccharomyces cerevisiae gene related to RNA processing: cloning of RNA1 and generation of a new allele with a novel phenotype. *Mol Cell Biol.* 5:907-15.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1992. Short Protocols in Molecular Biology. Greene Publishing Associates, New York.

Bailer, S.M., S. Siniossoglou, A. Podtelejnikov, A. Hellwig, M. Mann, and E. Hurt. 1998. Nup116p and nup100p are interchangeable through a conserved motif which constitutes a docking site for the mRNA transport factor gle2p. *Embo J.* 17:1107-19.

Bangs, P., B. Burke, C. Powers, R. Craig, A. Purohit, and S. Doxsey. 1998. Functional analysis of tpr: identification of nuclear pore complex association and nuclear localization domains and a role in mRNA export. *J Cell Biol.* 143:1801-12.

Bastos, R., N. Pante, and B. Burke. 1995. Nuclear pore complex proteins. *Int Rev Cytol.* 162B:257-302.

Bastos, R., A. Lin, M. Enarson, and B. Burke. 1996. Targeting and function in mRNA export of nuclear pore complex protein Nup153. *J Cell Biol.* 134:1141-56.

Bastos, R., L. Ribas de Pouplana, M. Enarson, K. Bodoor, and B. Burke. 1997. Nup84, a novel nucleoporin that is associated with CAN/Nup214 on the cytoplasmic face of the nuclear pore complex. *J Cell Biol.* 137:989-1000.

Becker, F., L. Block-Alper, G. Nakamura, J. Harada, K.D. Wittrup, and D.I. Meyer. 1999. Expression of the 180-kD ribosome receptor induces membrane proliferation and increased secretory activity in yeast. *JCB*. 146:273-284.

Bischoff, F.R., and H. Ponstingl. 1991. Mitotic regulator protein RCC1 is complexed with a nuclear ras-related polypeptide. *Proc. Natl. Acad. Sci. USA*. 88:10830-4.

Bischoff, F.R., H. Krebber, T. Kempf, I. Hermes, and H. Ponstingl. 1995. Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl. Acad. Sci. USA*. 92:1749-53.

Blobel, G. 1985. Gene gating: a hypothesis. *Proc Natl Acad Sci U S A*. 82:8527-9.

Bodoor, K., S. Shaikh, D. Salina, W.H. Raharjo, R. Bastos, M. Lohka, and B. Burke. 1999. Sequential recruitment of NPC proteins to the nuclear periphery at the end of mitosis. *J Cell Sci*. 112:2253-64.

Bogerd, A.M., J.A. Hoffman, D.C. Amberg, G.R. Fink, and L.I. Davis. 1994. nup1 mutants exhibit pleiotropic defects in nuclear pore complex function. *J Cell Biol.* 127:319-32.

Booth, J.W., K.D. Belanger, M.I. Sannella, and L.I. Davis. 1999. The yeast nucleoporin Nup2p is involved in nuclear export of importin alpha/Srp1p. *J Biol Chem*. 274:32360-7.

Bossie, M.A., C. DeHoratius, G. Barcelo, and P. Silver. 1992. A mutant nuclear protein with similarity to RNA binding proteins interferes with nuclear import in yeast. *Mol Biol Cell*. 3:875-93.

Bucci, M., and S.R. Wente. 1998. A novel fluorescence-based genetic strategy identifies mutants of Saccharomyces cerevisiae defective for nuclear pore complex assembly. *Mol Biol Cell*. 9:2439-61.

Byers, B. 1981. Cytology of the yeast cell cycle: Life Cycle and Inheritance. *In* Molecular Biology of the Yeast Saccharomyces cerevisiae. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York. 59-96.

Byers, B., and L. Goetsch. 1991. Preparation of yeast cell for thin-section electron microscopy. *Methods in Enzymology*. 194:602-608.

Carmo-Fonseca, M., and E.C. Hurt. 1991. Across the nuclear pores with the help of nucleoporins. *Chromosoma*. 101:199-205.

Chi, N.C., E.J. Adam, and S.A. Adam. 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J Cell Biol*. 130:265-74.

Chial, H.J., M.P. Rout, T.H. Giddings, and M. Winey. 1998. Saccharomyces cerevisiae Ndc1p is a shared component of nuclear pore complexes and spindle pole bodies. *J Cell Biol*. 143:1789-800.

Chow, T.Y.K., J.J. Ash, D. Dignard, and D.Y. Thomas. 1992. Screening and identification of a gene, PSE-1, that affects protein secretion in Saccharomyces cerevisiae. *J. Cell Sci.* 101:709-719.

Clark, K.L., M. Ohtsubo, T. Nishimoto, M. Goebl, and G.F. Sprague, Jr. 1991. The yeast SRM1 protein and human RCC1 protein share analogous functions. *Cell Regul.* 2:781-92.

Clegg, R.M. 1996. Fluorecence resonance energy transfer. *In* Fluorescence Imaging Spectroscopy and Microscopy. X.F. Wang and B. Herman, editors. Wiley, New York. 179-252.

Cole, C.N., and C.M. Hammell. 1998. Nucleocytoplasmic transport: driving and directing transport. *Curr Biol.* 8:R368-72.

Copeland, C.S., and M. Snyder. 1993. Nuclear pore complex antigens delineate nuclear envelope dynamics in vegetative and conjugating Saccharomyces cerevisiae. *Yeast.* 9:235-49.

Cordes, V.C., S. Reidenbach, H.R. Rackwitz, and W.W. Franke. 1997. Identification of protein p270/Tpr as a constitutive component of the nuclear pore complex-attached intranuclear filaments. *J Cell Biol.* 136:531-44.

Dabauvalle, M.C., R. Benavente, and N. Chaly. 1988. Monoclonal antibodies to a Mr 68,000 pore complex glycoprotein interfere with nuclear protein uptake in Xenopus oocytes. *Chromosoma.* 97:193-7.

Dabauvalle, M.C., K. Loos, and U. Scheer. 1990. Identification of a soluble precursor complex essential for nuclear pore assembly in vitro. *Chromosoma.* 100:56-66.

Damelin, M., and P.A. Silver. 2000. Mapping interactions between nuclear transport factors in living cells reveals pathways through the nuclear pore complex. *Mol Cell.* 5:133-40.

Davis, L.I., and G. Blobel. 1986. Identification and characterization of a nuclear pore complex protein. *Cell.* 45:699-709.

Davis, L.I., and G. Blobel. 1987. Nuclear pore complex contains a family of glycoproteins that includes p62: glycosylation through a previously unidentified cellular pathway. *Proc Natl Acad Sci U S A.* 84:7552-6.

De Vit, M.J., J.A. Waddle, and M. Johnston. 1997. Regulated nuclear translocation of the Mig1 glucose repressor. *Mol Biol Cell.* 8:1603-18.

DeHoratius, C., and P.A. Silver. 1996. Nuclear transport defects and nuclear envelope alterations are associated with mutation of the Saccharomyces cerevisiae NPL4 gene. *Mol Biol Cell.* 7:1835-55.

Dessev, G.N. 1992. Nuclear envelope structure. *Curr Opin Cell Biol.* 4:430-5.

Dingwall, C., and Laskey, R. A. 1991. Nuclear targeting sequences-a consensus? *Trends Biochem. Sci. 16*:478-481.

Dingwall, C., S. Kandels-Lewis, and B. Seraphin. 1995. A family of Ran binding proteins that includes nucleoporins. *Proc Natl Acad Sci U S A.* 92:7525-9.

Doye, V., R. Wepf, and E.C. Hurt. 1994. A novel nuclear pore protein Nup133p with distinct roles in poly(A)+ RNA transport and nuclear pore distribution. *Embo J.* 13:6062-75.

Doye, V., and E. Hurt. 1997. From nucleoporins to nuclear pore complexes. *Curr Opin Cell Biol*. 9:401-11.

Enarson, P., M. Enarson, R. Bastos, and B. Burke. 1998. Amino-terminal sequences that direct nucleoporin nup153 to the inner surface of the nuclear envelope. *Chromosoma*. 107:228-36.

Englmeier, L., J.C. Olivo, and I.W. Mattaj. 1999. Receptor-mediated substrate translocation through the nuclear pore complex without nucleotide triphosphate hydrolysis. *Curr Biol*. 9:30-41.

Fabre, E., W.C. Boelens, C. Wimmer, I.W. Mattaj, and E.C. Hurt. 1994. Nup145p is required for nuclear export of mRNA and binds homopolymeric RNA in vitro via a novel conserved motif. *Cell*. 78:275-89.

Fabre, E., and E.C. Hurt. 1994. Nuclear transport. *Curr Opin Cell Biol*. 6:335-42.

Fabre, E., N.L. Schlaich, and E.C. Hurt. 1995. Nucleocytoplasmic trafficking: what role for repeated motifs in nucleoporins? *Cold Spring Harb Symp Quant Biol*. 60:677-85.

Fabre, E., and E. Hurt. 1997. Yeast genetics to dissect the nuclear pore complex and nucleocytoplasmic trafficking. *Annu Rev Genet*. 31:277-313.

Fahrenkrog, B., E.C. Hurt, U. Aebi, and N. Pante. 1998. Molecular architecture of the yeast nuclear pore complex: localization of Nsp1p subcomplexes. *J Cell Biol*. 143:577-88.

Favreau, C., H.J. Worman, R.W. Wozniak, T. Frappier, and J.C. Courvalin. 1996. Cell cycle-dependent phosphorylation of nucleoporins and nuclear pore membrane protein Gp210. *Biochemistry*. 35:8035-44.

Featherstone, C., M.K. Darby, and L. Gerace. 1988. A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. *J Cell Biol*. 107:1289-97.

Feldherr, C.M., E. Kallenbach, and N. Schultz. 1984. Movement of a karyophilic protein through the nuclear pores of oocytes. *Nippon Ganka Gakkai Zasshi*. 88:1548-54.

Ferrigno, P., F. Posas, D. Koepp, H. Saito, and P.A. Silver. 1998. Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. *Embo J*. 17:5606-14.

Finlay, D.R., E. Meier, P. Bradley, J. Horecka, and D.J. Forbes. 1991. A complex of nuclear pore proteins required for pore function. *J Cell Biol*. 114:169-83.

Fischer, U., M. Michael, R. Luhrmann, and G. Dreyfuss. 1996. Signal-mediated nuclear export pathways of proteins and RNAs. *Trends Cell Biol*. 6:290-293.

Fontoura, B.M., G. Blobel, and M.J. Matunis. 1999. A conserved biogenesis pathway for nucleoporins: proteolytic processing of a 186-kilodalton precursor generates Nup98 and the novel nucleoporin, Nup96. *J Cell Biol*. 144:1097-112.

Fornerod, M., J. Boer, S. van Baal, M. Jaegle, M. von Lindern, K.G. Murti, D. Davis, J. Bonten, A. Buijs, and G. Grosveld. 1995. Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. *Oncogene*. 10:1739-48.

Fornerod, M., M. Ohno, M. Yoshida, and I.W. Mattaj. 1997a. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*. 90:1051-60.

Fornerod, M., J. van Deursen, S. van Baal, A. Reynolds, D. Davis, K.G. Murti, J. Fransen, and G. Grosveld. 1997b. The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88. *Embo J*. 16:807-16.

Gaits, F., G. Degols, K. Shiozaki, and P. Russell. 1998. Phosphorylation and association with the transcription factor Atf1 regulate localization of Spc1/Sty1 stress-activated kinase in fission yeast. *Genes Dev*. 12:1464-73.

Gant, T.M., M.W. Goldberg, and T.D. Allen. 1998. Nuclear envelope and nuclear pore assembly: analysis of assembly intermediates by electron microscopy. *Curr Opin Cell Biol*. 10:409-15.

Gerace, L. 1995. Nuclear export signals and the fast track to the cytoplasm. *Cell*. 82:341-4.

Giot, J.F., I. Mikaelian, M. Buisson, E. Manet, I. Joab, J.C. Nicolas, and A. Sergeant. 1991. Transcriptional interference between the EBV transcription factors EB1 and R: both DNA-binding and activation domains of EB1 are required. *Nucleic Acids Res*. 19:1251-8.

Goldberg, M.W., and T.D. Allen. 1995. Structural and functional organization of the nuclear envelope. *Curr Opin Cell Biol*. 7:301-9.

Goldberg, M.W., C. Wiese, T.D. Allen, and K.L. Wilson. 1997. Dimples, pores, star-rings, and thin rings on growing nuclear envelopes: evidence for structural intermediates in nuclear pore complex assembly. *J Cell Sci*. 110:409-20.

Goldfarb, D.S., J. Gariepy, G. Schoolnik, and R.D. Kornberg. 1986. Synthetic peptides as nuclear localization signals. *Cell*. 46:575-82.

Goldstein, A.L., C.A. Snay, C.V. Heath, and C.N. Cole. 1996. Pleiotropic nuclear defects associated with a conditional allele of the novel nucleoporin Rat9p/Nup85p. *Mol Biol Cell*. 7:917-34.

Gorlich, D., S. Prehn, R.A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*. 79:767-78.

Gorlich, D., S. Prehn, R.A. Laskey, and E. Hartmann. 1994. Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*. 79:767-78.

Gorlich, D., S. Kostka, R. Kraft, C. Dingwall, R.A. Laskey, E. Hartmann, and S. Prehn. 1995a. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr Biol*. 5:383-92.

Gorlich, D., F. Vogel, A.D. Mills, E. Hartmann, and R.A. Laskey. 1995b. Distinct functions for the two importin subunits in nuclear protein import. *Nature (London)*. 377:246-8.

Gorlich, D., and I.W. Mattaj. 1996. Nucleocytoplasmic transport. *Science*. 271:1513-8.

Gorlich, D., N. Pante, U. Kutay, U. Aebi, and F.R. Bischoff. 1996a. Identification of different roles for RanGDP and RanGTP in nuclear protein import. *Embo J.* 15:5584-94.

Gorlich, D., R. Kraft, S. Kostka, F. Vogel, E. Hartmann, R.A. Laskey, I.W. Mattaj, and E. Izaurraide. 1996b. Importin provides a link between nuclear protein import and U snRNA export. *Cell.* 87:21-32.

Gorlich, D., M. Dabrowski, F.R. Bischoff, U. Kutay, P. Bork, E. Hartmann, S. Prehn, and E. Izaurralde. 1997. A novel class of RanGTP binding proteins. *J. Cell Biol.* 138:65-80.

Gorsch, L.C., T.C. Dockendorff, and C.N. Cole. 1995. A conditional allele of the novel repeat-containing yeast nucleoporin RAT7/NUP159 causes both rapid cessation of mRNA export and reversible clustering of nuclear pore complexes. *J Cell Biol.* 129:939-55.

Grandi, P., V. Doye, and E.C. Hurt. 1993. Purification of NSP1 reveals complex formation with 'GLFG' nucleoporins and a novel nuclear pore protein NIC96. *Embo J.* 12:3061-71.

Grandi, P., N. Schlaich, H. Tekotte, and E.C. Hurt. 1995a. Functional interaction of Nic96p with a core nucleoporin complex consisting of Nsp1p, Nup49p and a novel protein Nup57p. *Embo J.* 14:76-87.

Grandi, P., S. Emig, C. Weise, F. Hucho, T. Pohl, and E.C. Hurt. 1995b. A novel nuclear pore protein Nup82p which specifically binds to a fraction of Nsp1p. *J Cell Biol.* 130:1263-73.

Grandi, P., T. Dang, N. Pane, A. Shevchenko, M. Mann, D. Forbes, and E. Hurt. 1997. Nup93, a vertebrate homologue of yeast Nic96p, forms a complex with a novel 205-kDa protein and is required for correct nuclear pore assembly. *Mol Biol Cell.* 8:2017-38.

Guan, T., S. Muller, G. Klier, N. Pante, J.M. Blevitt, M. Haner, B. Paschal, U. Aebi, and L. Gerace. 1995. Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex. *Mol Biol Cell.* 6:1591-603.

Hartwell, L.H. 1971. Genetic control of the cell division cycle in yeast. IV.genes contoling bud emergence and cytokenesis. *In* Exp. Cell Res. Vol. 69. 265-276.

Heath, C.V., C.S. Copeland, D.C. Amberg, V. Del Priore, M. Snyder, and C.N. Cole. 1995. Nuclear pore complex clustering and nuclear accumulation of poly(A)+ RNA associated with mutation of the Saccharomyces cerevisiae RAT2/NUP120 gene. *J Cell Biol.* 131:1677-97.

Herold, A., R. Truant, H. Wiegand, and B.R. Cullen. 1998. Determination of the functional domain organization of the importin alpha nuclear import factor. *J Cell Biol.* 143:309-18.

Ho, A.K., G.A. Raczniak, E.B. Ives, and S.R. Wente. 1998. The integral membrane protein snl1p is genetically linked to yeast nuclear pore complex function. *Mol Biol Cell.* 9:355-73.

Hood, J.K., and P.A. Silver. 1998. Cse1p is required for export of Srp1p/Importin-alpha from the nucleus in saccharomyces cerevisiae. *J Biol Chem.* 273:35142-6.

Hood, J.K., and P.A. Silver. 1999. In or out? Regulating nuclear transprot. Cuu. opin. Cell Biol. 11:241-247.

Hopper, A.K., H.M. Traglia, and R.W. Dunst. 1990. The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. *J Cell Biol.* 111;309-21.

Hu, T., T. Guan, and L. Gerace. 1996. Molecular and functional characterization of the p62 complex, an assembly of nuclear pore complex glycoproteins. *J Cell Biol.* 134:589-601.

Imamoto, N., T. Shimamoto, S. Kose, T. Takao, T. Tachibana, M. Matsubae, T. Sekimoto, Y. Shimonishi, and Y. Yoneda. 1995a. The nuclear pore-targeting complex binds to nuclear pores after association with a karyophile. *FEBS Lett.* 368:415-9.

Imamoto, N., T. Tachibana, M. Matsubae, and Y. Yoneda. 1995b. A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.* 270:8559-65.

Iovine, M.K., J.L. Watkins, and S.R. Wente. 1995. The GLFG repetitive region of the nucleoporin Nup116p interacts with Kap95p, an essential yeast nuclear import factor. *J Cell Biol.* 131:1699-713.

Iovine, M.K., and S.R. Wente. 1997. A nuclear export signal in Kap95p is required for both recycling the import factor and interaction with the nucleoporin GLFG repeat regions of Nup116p and Nup100p. *J. Cell Biol.* 137:797-811.

Izaurralde, E., J. Lewis, C. Gamberi, A. Jarmolowski, C. McGuigan, and I.W. Mattaj. 1995. A cap-binding protein complex mediating U snRNA export. *Nature.* 376:709-12.

Jackson, C.L., and J.E. Casanova. 2000. Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol.* 10:60-7.

Jarmolowski, A., W.C. Boelens, E. Izaurralde, and I.W. Mattaj. 1994. Nuclear export of different classes of RNA is mediated by specific factors. *J Cell Biol.* 124:627-35.

Kadowaki, T., Y. Zhao, and A.M. Tartakoff. 1992. A conditional yeast mutant deficient in mRNA transport from nucleus to cytoplasm. *Proc Natl Acad Sci U S A.* 89:2312-6.

Kaffman, A., N.M. Rank, and E.K. O'Shea. 1998a. Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. *Genes Dev.* 12:2673-83.

Kaffman, A., N.M. Rank, E.M. O'Neill, L.S. Huang, and E.K. O'Shea. 1998b. The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature.* 396:482-6.

Kehlenbach, R.H., A. Dickmanns, A. Kehlenbach, T. Guan, and L. Gerace. 1999. A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. *J Cell Biol.* 145:645-57.

Kenna, M.A., J.G. Petranka, J.L. Reilly, and L.I. Davis. 1996. Yeast N1e3p/Nup170p is required for normal stoichiometry of FG nucleoporins within the nuclear pore complex. *Mol Cell Biol.* 16:2025-36.

Kilmartin, J.V., and A.E. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast *Saccharomyces*. *J. Cell Biol.* 98:922-33.

Kita, K., S. Omata, and T. Horigome. 1993. Purification and characterization of a nuclear pore glycoprotein complex containing p62. *J Biochem (Tokyo)*. 113:377-82.

Kosova, B., N. Pante, C. Rollenhagen, A. Podtelejnikov, M. Mann, U. Aebi, and E. Hurt. 2000. Mlp2p, a component of nuclear pore attached intranuclear filaments, associates with nic96p. *J Biol Chem*. 275:343-50.

Kraemer, D., R.W. Wozniak, G. Blobel, and A. Radu. 1994. The human CAN protein, a putative oncogene product associated with myeloid leukemogenesis, is a nuclear pore complex protein that faces the cytoplasm. *Proc Natl Acad Sci U S A*. 91:1519-23.

Kraemer, D.M., C. Strambio-de-Castillia, G. Blobel, and M.P. Rout. 1995. The essential yeast nucleoporin NUP159 is located on the cytoplasmic side of the nuclear pore complex and serves in karyopherin-mediated binding of transport substrate. *J Biol Chem*. 270:19017-21.

Kraemer, D., and G. Blobel. 1997. mRNA binding protein mrnp 41 localizes to both nucleus and cytoplasm. *Proc Natl Acad Sci U S A*. 94:9119-24.

Kranz, J.E., and C. Holm. 1990. Cloning by function: an alternative approach for identifying yeast homologs of genes from other organisms. *Proc Natl Acad Sci U S A*. 87:6629-33.

Kuge, S., N. Jones, and A. Nomoto. 1997. Regulation of yAP-1 nuclear localization in response to oxidative stress. *Embo J*. 16:1710-20.

Kunzler, M., and E.C. Hurt. 1998. Cse1p functions as the nuclear export receptor for importin alpha in yeast. *FEBS Lett*. 433:185-90.

Kutay, U., F.R. Bischoff, S. Kostka, R. Kraft, and D. Gorlich. 1997. Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell*. 90:1061-71.

Kutay, U., G. Lipowsky, E. Izaurralde, F.R. Bischoff, P. Schwarzmaier, E. Hartmann, and D. Gorlich. 1998. Identification of a tRNA-specific nuclear export receptor. *Mol Cell*. 1:359-69.

Li, O., C.V. Heath, D.C. Amberg, T.C. Dockendorff, C.S. Copeland, M. Snyder, and C.N. Cole. 1995. Mutation or deletion of the Saccharomyces cerevisiae RAT3/NUP133 gene causes temperature-dependent nuclear accumulation of poly(A)+ RNA and constitutive clustering of nuclear pore complexes. *Mol Biol Cell*. 6:401-417.

Macaulay, C., E. Meier, and D.J. Forbes. 1995. Differential mitotic phosphorylation of proteins of the nuclear pore complex. *J Biol Chem*. 270:254-62.

Macaulay, C., and D.J. Forbes. 1996. Assembly of the nuclear pore: biochemically distinct steps revealed with NEM, GTP gamma S, and BAPTA. *J Cell Biol*. 132:5-20.

Mahajan, R., C. Delphin, T. Guan, L. Gerace, and F. Melchior. 1997. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell*. 88:97-107.

Mahajan, R., L. Gerace, and F. Melchior. 1998. Molecular characterization of the SUMO-1 modification of RanGAP1 and its role in nuclear envelope association. *J. Cell Biol.* 140:259-70.

Marelli, M., J.D. Aitchison, and R.W. Wozniak. 1998. Specific binding of the karyopherin kap121p to a subunit of the nuclear pore complex containing nup53p, nup59p, and nup170p. *J Cell Biol.* 143:1813-30.

Mattaj, I.W., and L. Englmeier. 1998. Nucleocytoplasmic transport: the soluble phase. *J Cell Biol.* 143:23-34.

Matunis, M.J., J. Wu, and G. Blobel. 1998. SUMO-1 modification and its role in targeting the ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. *J. Cell Biol.* 140:499-509.

Meier, U.T., and G. Blobel. 1992. Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell.* 70:127-38.

Melchior, F., B. Paschal, J. Evans, and L. Gerace. 1993. Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J Cell Biol.* 123:1649-59.

Melchior, F., and L. Gerace. 1998. Two-way trafficking with Ran. *Trends Cell Biol.* 8:175-9.

Moore, M.S., and G. Blobel. 1993. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature.* 365:661-3.

Moore, M.S., and G. Blobel. 1994. Purification of a Ran-interacting protein that is required for protein import into the nucleus. *Proc Natl Acad Sci U S A.* 91:10212-6.

Moroianu, J., Hijikata, M., Blobel, G., and Radu, A. 1995. Mammalian karyopherin alpha 1 beta and alpha 2 beta heterodimers: alpha 1 or alpha 2 subunit binds nuclear localization signal and beta subunit interacts with peptide repeat-containing nucleoporins. *Proc Natl Acad Sci U S A.* 92:6532-6.

Moroianu, J., G. Blobel, and A. Radu. 1996. Nuclear protein import: Ran-GTP dissociates the karyopherin alphabeta heterodimer by displacing alpha from an overlapping binding site on beta. *Proc Natl Acad Sci U S A.* 93:7059-62.

Moroianu, J., G. Blobel, and A. Radu. 1997. RanGTP-mediated nuclear export of karyopherin alpha involves its interaction with the nucleoporin Nup153. *Proc Natl Acad Sci U S A.* 94:9699-704.

Murphy, R., J.L. Watkins, and S.R. Wente. 1996. GLE2, a Saccharomyces cerevisiae homologue of the Schizosaccharomyces pombe export factor RAE1, is required for nuclear pore complex structure and function. *Mol Biol Cell.* 7:1921-37.

Murphy, R., and S.R. Wente. 1996. An RNA-export mediator with an essential nuclear export signal. *Nature.* 383:357-60.

Mutvei, A., S. Dihlmann, W. Herth, and E.C. Hurt. 1992. NSP1 depletion in yeast affects nuclear pore formation and nuclear accumulation. *Eur. J. Cell Biol.* 59:280-295.

Nakielny, S., and G. Dreyfuss. 1999. Transport of proteins and RNAs in and out of the nucleus. *Cell*. 99:677-90.

Nakielny, S., S. Shaikh, B. Burke, and G. Dreyfuss. 1999. Nup153 is an M9-containing mobile nucleoporin with a novel Ran-binding domain. *Embo J*. 18:1982-95.

Nehrbass, U., and G. Blobel. 1996. Role of the nuclear transport factor p10 in nuclear import. *Science*. 272:120-2.

Nehrbass, U., M.P. Rout, S. Maguire, G. Blobel, and R.W. Wozniak. 1996. The yeast nucleoporin Nup188p interacts genetically and physically with the core structures of the nuclear pore complex. *J Cell Biol*. 133:1153-62.

Neville, M., F. Stutz, L. Lee, L.I. Davis, and M. Rosbash. 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr Biol*. 7:767-75.

Newmeyer, D.D., D.R. Finlay, and D.J. Forbes. 1986. In vitro transport of a fluorescent nuclear protein and exclusion of non-nuclear proteins. *Yen Ko Hsueh Pao*. 2:211-4.

Niedenthal, R.K., L. Riles, M. Johnston, and J.H. Hegemann. 1996. Green fluorescent Protein as a marker for gene expression and subcellular localization in budding Yeast. *Yeast*. 12:773-786.

Nuttley, W.M., A.M. Brade, C. Gaillardin, G.A. Eitzen, J.R. Glover, J.D. Aitchison, and R.A. Rachubinski. 1994. Pay4 a gene required for peroxisome assembly in the yeast Yarrowia lipolytica, encodes a novel member of a family of ATPases. *J Cell Biol*. 269:556-66

Ohtsubo, M., H. Okazaki, and T. Nishimoto. 1989. The RCC1 protein, a regulator for the onset of chromosome condensation locates in the nucleus and binds to DNA. *J Cell Biol*. 109:1389-97.

Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont. 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science*. 278:141-4.

Paine, P.L., L.C. Moore, and S.B. Horowitz. 1975. Nuclear envelope permeability. *Nature*. 254:109-14.

Pante, N., R. Bastos, I. McMorrow, B. Burke, and U. Aebi. 1994. Interactions and three-dimensional localization of a group of nuclear pore complex proteins. *J Cell Biol*. 126:603-17.

Pante, N., and U. Aebi. 1996. Sequential binding of import ligands to distinct nucleopore regions during their nuclear import. *Science*. 273:1729-32.

Parrish, M.L., C. Sengstag, J.D. Rine, and R.L. Wright. 1995. Identification of the sequences in HMG1-CoA reductase required for karmellae assmebly. *Mol. Cell Biol*. 11:1535-47.

Paschal, B.M., and L. Gerace. 1995. Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62. *J Cell Biol*. 129:925-37.

Pemberton, L.F., M.P. Rout, and G. Blobel. 1995. Disruption of the nucleoporin gene NUP133 results in clustering of nuclear pore complexes. *Proc Natl Acad Sci U S A*. 92:1187-91.

Pemberton, L.F., J.S. Rosenblum, and G. Blobel. 1997. A distinct and parallel pathway for the nuclear import of an mRNA- binding protein. *J. Cell Biol.* 139:1645-53.

Pemberton, L.F., G. Blobel, and J.S. Rosenblum. 1998. Transport routes through the nuclear pore complex. *Curr Opin Cell Biol.* 10:392-9.

Percipalle, P., W.D. Clarkson, H.M. Kent, D. Rhodes, and M. Stewart. 1997. Molecular interactions between the importin alpha/beta heterodimer and proteins involved in vertebrate nuclear protein import. *J Mol Biol.* 266:722-32.

Powers, M.A., C. Macaulay, F.R. Masiarz, and D.J. Forbes. 1995. Reconstituted nuclei depleted of a vertebrate GLFG nuclear pore protein, p97, import but are defective in nuclear growth and replication. *J Cell Biol.* 128:721-36.

Pringle, J.R., and L.H. Hartwell. 1981. The Saccharomyces cerevisae cell cycle. *In* The Molecular Biology of the yeast Saccharomyces cervisae: Life Cycle and inheritance. Cold Spring Harbor Laboratory., Cold Spring Harbor, New York, NY. 97-149.

Radu, A., G. Blobel, and R.W. Wozniak. 1993. Nup155 is a novel nuclear pore complex protein that contains neither repetitive sequence motifs nor reacts with WGA. *J Cell Biol.* 121:1-9.

Radu, A., G. Blobel, and R.W. Wozniak. 1994. Nup107 is a novel nuclear pore complex protein that contains a leucine zipper. *J Biol Chem.* 269:17600-5.

Radu, A., G. Blobel, and M.S. Moore. 1995a. Identification of a protein complex that is required for nuclear prote...in import and mediates docking of import substrate to distinct nucleoporins. *Proc Natl Acad Sci U S A.* 92:1769-73.

Radu, A., M.S. Moore, and G. Blobel. 1995b. The peptide repeat domain of nucleoporin Nup98 functions as a docking site in trasnport across the nuclear pore complex. *Cell.* 81:215-222.

Reichelt, R., A. Holzenburg, E.L. Buhle, Jr., M. Jarnik, A. Engel, and U. Aebi. 1990. Correlation between structure and mass distribution of the nuclear pore complex and of distinct pore complex components. *J Cell Biol.* 110:883-94.

Rexach, M., and G. Blobel. 1995. Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell.* 83:683-92.

Ribbeck, K., G. Lipowsky, H.M. Kent, M. Stewart, and D. Gorlich. 1998. NTF2 mediates nuclear import of Ran. *Embo J.* 17:6587-98.

Ribbeck, K., U. Kutay, E. Paraskeva, and D. G#rlich. 1999. The translocation of transportin-cargo complexes through nuclear pores is independent of both Ran and energy. *Curr Biol.* 9:47-50.

Richards, S.A., K.M. Lounsbury, K.L. Carey, and I.G. Macara. 1996. A nuclear export signal is essential for the cytosolic localization of the Ran binding protein, RanBP1. *J. Cell Biol.* 134:1157-68.

Richards, S.A., K.L. Carey, and I.G. Macara. 1997. Requirement of guanosine triphosphate-bound ran for signal-mediated nuclear protein export. *Science.* 276:1842-4.

Richardson, W.D., A.D. Mills, S.M. Dilworth, R.A. Laskey, and C. Dingwall. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell*. 52:655-64.

Rosenblum, J.S., L.F. Pemberton, and G. Blobel. 1997. A nuclear import pathway for a protein involved in tRNA maturation. *J. Cell Biol.* 139:1655-61.

Rothstein, R. 1990. Targeting, disruption, replacement and allele rescue: integrative transformation in Yeast. *Methods Enzymol.* 194:281-301.

Rout, M.P., and J.V. Kilmartin. 1990. Components of the yeast spindle and spindle pole body. *J Cell Biol*. 111:1913-27.

Rout, M.P., and S.R. Wente. 1994. Pores for thought: nuclear pore proteins. *Trends Cell Biol.* 4:357-365.

Rout, M.P., G. Blobel, and J.D. Aitchison. 1997. A distinct nuclear import pathway used by ribosomal proteins. *Cell*. 89:715-25.

Rout, M.P., J.D. Aitchison, A. Suprapto, K. Hjertaas, Y. Zhao, and B.T. Chait. 2000. The yeast nuclear pore complex: composition, architecture, and transport mechanism. *J Cell Biol*. 148:635-51.

Sarkar, S., and A.K. Hopper. 1998. tRNA nuclear export in saccharomyces cerevisiae: In situ hybridization analysis. *Mol Biol Cell*. 9:3041-55.

Schlaich, N.L., M. Haner, A. Lustig, U. Aebi, and E.C. Hurt. 1997. In vitro reconstitution of a heterotrimeric nucleoporin complex consisting of recombinant Nsp1p, Nup49p, and Nup57p. *Mol Biol Cell*. 8:33-46.

Schlenstedt, G., E. Smirnova, R. Deane, J. Solsbacher, U. Kutay, D. Gorlich, H. Ponstingl, and F.R. Bischoff. 1997. Yrb4p, a yeast ran-GTP-binding protein involved in import of ribosomal protein L25 into the nucleus. *Embo J.* 16:6237-49.

Schneiter, R., M. Hitomi, A.S. Ivessa, E.V. Fasch, S.D. Kohlwein, and A.M. Tartakoff. 1996. A yeast acetyl coenzyme A carboxylase mutant links very-long-chain fatty acid synthesis to the structure and function of the nuclear membrane-pore complex. *Mol Cell Biol*. 16:7161-72.

Schwoebel, E.D., B. Talcott, I. Cushman, and M.S. Moore. 1998. Ran-dependent signal-mediated nuclear import does not require GTP hydrolysis by Ran. *J Biol Chem*. 273:35170-5.

Seedorf, M., and P.A. Silver. 1997. Importin/karyopherin protein family members required for mRNA export from the nucleus. *Proc Natl Acad Sci U S A*. 94:8590-5.

Seedorf, M., M. Damelin, J. Kahana, T. Taura, and P.A. Silver. 1999. Interactions between a Nuclear Transporter and a Subset of Nuclear Pore Complex Proteins Depend on Ran GTPase. *Mol Cell Biol*. 19:1547-1557.

Shah, S., S. Tugendreich, and D. Forbes. 1998. Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr. *J Cell Biol*. 141:31-49.

Shah, S., and D.J. Forbes. 1998. Separate nuclear import pathways converge on the nucleoporin Nup153 and can be dissected with dominant-negative inhibitors. *Curr Biol.* 31;8:1376-1386.

Shan, X., Z. Xue, G. Euskirchen, and T. Melese. 1997. NNF1 is an essential yeast gene required for proper spindle orientation, nucleolar and nuclear envelope structure and mRNA export. *J Cell Sci.* 110:1615-24.

Sherman, F., G.R. Fink, and J.B. Hicks. 1986. Methods in Yeast Genetics. *Cold Spring Harbor Laboratory, Cold Spring Harbor*, New York. 186 pp.

Shulga, N., P. Roberts, Z. Gu, L. Spitz, M.M. Tabb, M. Nomura, and D.S. Goldfarb. 1996. In vivo nuclear transport kinetics in Saccharomyces cerevisiae: a role for heat shock protein 70 during targeting and translocation. *J Cell Biol.* 135:329-39.

Shulga, N., N. Mosammaparast, R. Wozniak, and D.S. Goldfarb. 2000. Yeast nucleoporins involved in passive nuclear envelope permeability. *J Cell Biol.* 149:1027-38.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics.* 122:19-27.

Siniossoglou, S., C. Wimmer, M. Rieger, V. Doye, H. Tekotte, C. Weise, S. Emig, A. Segref, and E.C. Hurt. 1996. A novel complex of nucleoporins, which includes Sec13p and a Sec13p homolog, is essential for normal nuclear pores. *Cell.* 84:265-75.

Siniossoglou, S., H. Santos-Rosa, J. Rappsilber, M. Mann, and E. Hurt. 1998. A novel complex of membrane proteins required for formation of a spherical nucleus. *Embo J.* 17:6449-64.

Smith, S., and G. Blobel. 1994. Colocalization of vertebrate lamin B and lamin B receptor (LBR) in nuclear envelopes and in LBR-induced membrane stacks of the yeast *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA*. 91:10124-10128.

Smith, A., A. Brownawell, and I.G. Macara. 1998. Nuclear import of Ran is mediated by the transport factor NTF2. *Curr Biol.* 31;8:1403-1406.

Snow, C.M., A. Senior, and L. Gerace. 1987. Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. *J Cell Biol.* 104:1143-56.

Solsbacher, J., P. Maurer, F.R. Bischoff, and G. Schlenstedt. 1998. Cse1p is involved in export of yeast importin alpha from the nucleus. *Mol Cell Biol.* 18:6805-15.

Stade, K., C.S. Ford, C. Guthrie, and K. Weis. 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell.* 90:1041-50.

Stoffler, D., B. Fahrenkrog, and U. Aebi. 1999. The nuclear pore complex: from molecular architecture to functional dynamics. *Curr Opin Cell Biol.* 11:391-401.

Strahm, Y., B. Fahrenkrog, D. Zenklusen, E. Rychner, J. Kantor, M. Rosbach, and F. Stutz. 1999. The RNA export factor Gle1p is located on the cytoplasmic fibrils of the NPC and physically interacts with the FG-nucleoporin Rip1p, the DEAD-box protein Rat8p/Dbp5p and a new protein Ymr 255p. *Embo J.* 18:5761-77.

Strambio-de-Castillia, C., G. Blobel, and M.P. Rout. 1995. Isolation and characterization of nuclear envelopes from the Yeast *Sacchatomyces*. *J. Cell. Biol.* 131:19-31.

Stukenberg, P.T., K.D. Lustig, T.J. McGarry, R.W. King, J. Kuang, and M.W. Kirschner. 1997. Systematic identification of mitotic phosphoproteins. *Curr. Biol.* 7:338-348.

Sukegawa, J., and G. Blobel. 1993. A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell.* 72:29-38.

Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth. 1993. destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. *EMBO J.* 12:1969-1978.

Talcott, B., and M.S. Moore. 1999. Getting across the nuclear pore complex. *Trends Cell Biol.* 9:312-8.

Tcheperegine, S.E., M. Marelli, and R.W. Wozniak. 1999. Topology and functional domains of the yeast pore membrane protein Pom152p. *J Biol Chem.* 274:5252-8.

Toone, W.M., S. Kuge, M. Samuels, B.A. Morgan, T. Toda, and N. Jones. 1998. Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. *Genes Dev.* 12:1453-63.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

Uetz, P., L. Giot, G. Cagney, T.A. Mansfield, R.S. Judson, J.R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields, and J.M. Rothberg. 2000. A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. *Nature.* 403:623-7.

van Deursen, J., J. Boer, L. Kasper, and G. Grosveld. 1996. G2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene CAN/Nup214. *Embo J.* 15:5574-83.

Vergeres, G., J. Ramsden, L. Waskell. 1995. The carboxyl termiuns of the membrane binding domain of cytochrome b5 spans the bilayer of the endoplasmic reticulum. *J Biol. Chem.* 270:3414-22.

Weis, K., I.W. Mattaj, and A.I. Lamond. 1995. Identification of hSRP1 alpha as a functional receptor for nuclear localization sequences. *Science.* 268:1049-53.

Wente, S.R., M.P. Rout, and G. Blobel. 1992. A new family of yeast nuclear pore complex proteins. *J Cell Biol.* 119:705-23.

Wente, S.R., and G. Blobel. 1993. A temperature-sensitive NUP116 null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleocytoplasmic traffic. *J Cell Biol.* 123:275-84.

Wente, S.R., and G. Blobel. 1994. NUP145 encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure. *J Cell Biol.* 125:955-69.

Winey, M., D. Yarar, T.H. Giddings, Jr., and D.N. Mastronarde. 1997. Nuclear pore complex number and distribution throughout the Saccharomyces cerevisiae cell cycle by three-dimensional reconstruction from electron micrographs of nuclear envelopes. *Mol Biol Cell*. 8:2119-32.

Wozniak, R.W., G. Blobel, and M.P. Rout. 1994. POM152 is an integral membrane protein of the pore membrane domain of the nuclear envelope. *J. Cell Biol.* 125:31-42.

Wozniak, R.W., M.P. Rout, and J.D. Aitchison. 1998. Karyopherins and kissing cousins. *Trends Cell Biol*. 8:184-8.

Wray, B.E., and R. Sealock. 1984. Ultrastructural immunocytochemistry of particulate fractions using polyvinyl chloride microculture wells. *J Histochem Cytochem*. 32:1117-20.

Wright, R., M. Basson, L. D'Ari, and J. Rine. 1988. Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs sorrunding the yeast nucleus. *J. Cell Biol.* 107:101-114.

Wright, R., M. Basson, L. D'Ari, and J. Rine. 1988. Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs sorrunding the yeast nucleus. *J. Cell Biol.* 107:101-114.

Wu, J., M.J. Matunis, D. Kraemer, G. Blobel, and E. Coutavas. 1995. Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran- GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J Biol Chem*. 270:14209-13.

Yan, C., N. Leibowitz, and T. Melese. 1997. A role for the divergent actin gene, ACT2, in nuclear pore structure and function. *Embo J*. 16:3572-86.

Yan, C., L.H. Lee, and L.I. Davis. 1998. Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. *Embo J*. 17:7416-7429.

Yang, Q., M.P. Rout, and C.W. Akey. 1998. Three-dimensional architecture of the isolated yeast nuclear pore complex: functional and evolutionary implications. *Mol Cell*. 1:519-29.

Yaseen, N.R., and G. Blobel. 1997. Cloning and characterization of human karyopherin beta3. *Proc Natl Acad Sci U S A*. 94:4451-6.

Yokoyama, N., N. Hayashi, T. Seki, N. Pante, T. Ohba, K. Nishii, K. Kuma, T. Hayashida, T. Miyata, U. Aebi, and et al. 1995. A giant nucleopore protein that binds Ran/TC4. *Nature*. 376:184-8.

Zabel, U., V. Doye, H. Tekotte, R. Wepf, P. Grandi, and E.C. Hurt. 1996. Nic96p is required for nuclear pore formation and functionally interacts with a novel nucleoporin, Nup188p. *J Cell Biol*. 133:1141-52.

Zasloff, M. 1983. tRNA transport from the nucleus in a eukaryotic cell: carrier-mediated translocation process. *Proc Natl Acad Sci U S A*. 80:6436-40.

Zimowska, G., J.P. Aris, and M.R. Paddy. 1997. A Drosophila Tpr protein homolog is localized both in the extrachromosomal channel network and to nuclear pore complexes.

Kaibogaku Zasshi. 72:123-33.